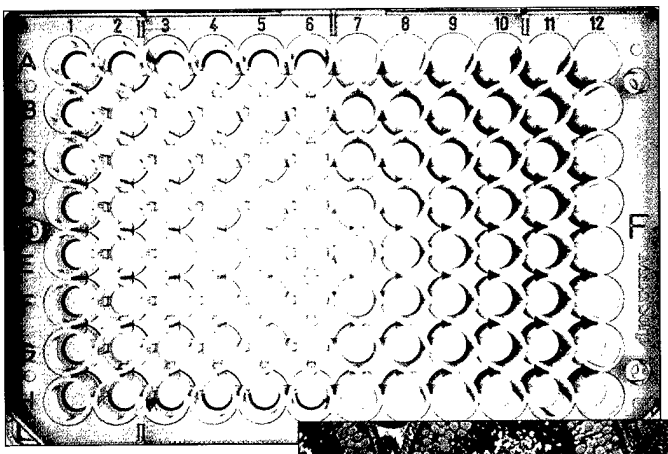
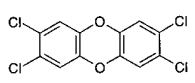
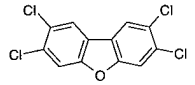
Biomonitoring of Environmental Status and Trends (BEST) Program: Selected Methods for Monitoring Chemical Contaminants and their Effects in Aquatic Ecosystems

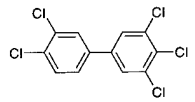




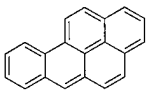
(a) 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin



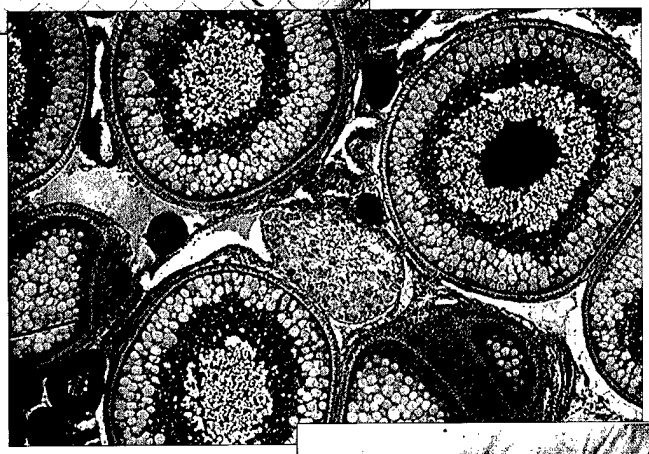
(b) 2,3,7,8-Tetrachlorodibenzofuran



(c) 3,3´,4,4´,5-Pentachlorobiphenyl (PCB 126)



(d) Benzo[a]pyrene



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Biomonitoring of Environmental Status and Trends (BEST) Program: Selected Methods for Monitoring Chemical Contaminants and their Effects in Aquatic Ecosystems

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Biomonitoring of Environmental Status and Trends (BEST) Program: Selected Methods for Monitoring Chemical Contaminants and their Effects in Aquatic Ecosystems

Christopher J. Schmitt and Gail M. Dethloff, editors

Abstract: This document describes the suite of biological methods of the U.S. Geological Survey-Biomonitoring of Environmental Status and Trends program for monitoring chemical contaminants and their effects on fish. The methods, which were selected by panels of experts, are being field-tested in rivers of the Mississippi River, Columbia River, and Rio Grande basins. General health biomarkers include a health assessment index based on gross observation; histopathological examination of selected organs and tissues; condition factor; and the heptosomatic and splenosomatic indices. Immune system indicators are plasma lysozyme activity and measures of splenic macrophage aggregates. Reproductive biomarkers include plasma concentrations of sex steroid hormones (17β-estradiol and 11-ketotestosterone) and vitellogenin, gonadal histopathology (including reproductive stage and, in females, gonadal atresia), and the gonadosomatic index. Indicators of exposure to polycyclic aromatic and polyhalogenated hydrocarbons are the H4IIE rat hepatoma cell bioassay (performed on solvent extracts of composite fish samples) and hepatic ethoxyresorufin-*O*-deethylase activity. Stable nitrogen isotope ratios are used to assess the trophic position of the fish and their exposure to sewage and other animal wastes. For each indicator we describe endpoint(s) and methods, and discuss the indicator's value and limitations for contaminant monitoring and assessment.

Keywords: Contaminants, biomarkers, fish health, aromatic hydrocarbons, histopathology, atresia, sex steroid hormones, 17B-estradiol, 11-ketotestosterone, vitellogenin, GSI, HSI, SSI, HAI, EROD, H4IIE, condition factor, lysozyme, macrophage aggregates, nitrogen isotope ratios.

PREFACE

The Biomonitoring of Environmental Status and Trends (BEST) program was initiated in 1991 by the U.S. Fish and Wildlife Service (FWS) as a revision and expansion of the National Contaminant Biomonitoring Program (NCBP). The NCBP originated as part of the National Pesticide Monitoring Program (NPMP), a multi-agency effort through which temporal and geographic trends in persistent contaminant concentrations were documented through the collection and chemical analysis of environmental media. The FWS participated in the NPMP by collecting and analyzing freshwater fish and European starlings *(Sturnus vulgaris)* from nationwide networks of stations and by analyzing the wings of hunter-killed ducks from the four major U.S. flyways (Johnson et al. 1967). In response to a growing need to address new classes of chemicals for which the chemical analysis of animal tissues was not an effective monitoring method, and to focus its activities on biological resources rather than on chemicals, the FWS re-named its monitoring program (to NCBP) in the anticipation of adding biological monitoring and assessment components targeted towards lands and species of concern to the agency. To a great extent, the BEST program is the result of FWS planning for this expanded monitoring. In 1993, the BEST program, as well as curatorial responsibility for the historic NCBP data bases and sample archives, were transferred to the National Biological Service (BEST 1996). The NBS became the Biological Resources Division of the U. S. Geological Survey in 1996. Appendix 1 traces the development of the BEST program in detail.

To test the feasibility of implementing selected components of the BEST program at the national and regional scales, pilot projects based on the historic NCBP network of sampling stations were designed and initiated in 1995 and 1997 (Schmitt et al. 1995; Bartish et al. 1997). This report summarizes the scope and intent of the projects relative to the continuing development of the BEST program and the monitoring methods being evaluated.

THE 1995 AND 1997 BEST PROJECTS

Christopher J. Schmitt, Donald E. Tillitt and Vicki S. Blazer

The National Contaminant Biomonitoring Program (NCBP) fish network was last fully sampled and analyzed in 1986 (Schmitt et al. 1999). Due to continued interest in bioaccumulable contaminants, the BEST program proposed to update selected parts of the NCBP database, beginning with the Mississippi River basin, in the fall of 1995 (Schmitt et al. 1995). These investigations would also serve as platforms for demonstrating and further evaluating some of the biomarkers and bioassays proposed for use in the program in a habitat class identified as important to the BEST program — freshwater, flowing, large river (BEST 1996) — at a large scale. The 1995 study also included sites on smaller rivers and streams in selected basins sampled by the National Water Quality Assessment (NAWQA) program of the U.S. Geological Survey-Water Resources Division (USGS-WRD). A second round of sampling was initiated in 1997 in the Rio Grande and Columbia River basins (Bartish et al. 1997), in cooperation with the National Stream Quantity Accounting Network (NASQAN II) program of the USGS-WRD (Hooper et al. 1996). The specific objectives of the 1995 and 1997 studies were to:

(1) Document and characterize the geographic distribution of chemical contaminants and their effects on fish and wildlife in the large rivers of the Mississippi, Columbia, and Rio Grande basins, and compare the findings for bioaccumulable contaminants with those of previous NCBP fish collections.

(2) Field test, evaluate, and optimize aquatic indicators selected for use in the BEST program.

(3) Evaluate and demonstrate the technical and logistic feasibility of implementing the BEST program through partnerships with science centers, cooperative research units, universities, and other monitoring programs and Department of the Interior agencies.

(4) Evaluate the compatibility of selected components of the BEST program with the NAWQA and NASQAN-II programs of the USGS-WRD.

Site Selection

The 1995 and 1997 projects were developed with the cooperation of the NAWQA and NASQAN II programs of the USGS-WRD because these programs collect a wide range of water quality and hydrologic data. The NAWQA program also collects information on land use and ecological conditions that may be useful for evaluating the utility of the biomarkers selected for use in the BEST program and for interpreting the results of the investigations. For 1995, the 38 NCBP stations located in the Mississippi River drainage were targeted for sampling. These stations are affected by a wide range of agricultural, industrial, mining, and urban pollutants (Goolsby 1996). In addition, the stations in the Mississippi basin were most in need of updated information because extensive flooding in the Midwest during 1993 and 1995 greatly redistributed contaminants since the last NCBP collection in 1986 (Rostad 1997). For budgetary reasons, three targeted sites were eliminated from consideration — two in the upper Platte River system, which had been sampled recently by the NAWQA program (Goodbred et al. 1997; Tate and Heiny 1996; Heiny and Tate 1997); and one on the Upper Missouri that had historically yielded cold-water fishes, a guild not targeted by our study. To integrate the study with ongoing NAWQA investigations and thereby expand it into more habitats, NAWQA sites on lower-order streams in smaller watersheds within the Mississippi basin were also included. A reference site (water supply reservoirs of the USGS Leetown Science Center in Kearneysville, WV) was included. The final list of 1995 stations comprised 35 former NCBP stations, all but one of which were sampled; a total of 13 NAWQA sites in two study units — four in the Eastern Iowa Basins (Kalkhoff 1994) and nine in the Mississippi Embayment (Mallory 1994) Study Units; and the reference site (Fig. 1).

The 1997 study represented an extension of the 1995 project, with the added specific objective of testing the compatibility of the protocol and methods evaluated in the 1995 Mississippi basin project with the re-design of the NASQAN program (NASQAN II; Hooper et al. 1996). The NASQAN II program monitors dissolved and suspended concentrations of many water constituents (elemental contaminants, hydrophilic pesticides, nutrients, etc.) in four large river systems — the Mississippi, Columbia, Colorado, and Rio Grande. The information on bioaccumulable contaminants and contaminant effects generated by the BEST program therefore complemented the chemical measurements of the NASQAN II program.

Consequently, the 1997 project was also considered a pilot for a possible national-scale combined network for large rivers. Stations selected for sampling in 1997 included ten historic NCBP stations and four NASQAN II stations in the Columbia basin; and four NCBP and six NASQAN II stations in the Rio Grande basin. An additional site in the Columbia basin (Station 506) was added to provide collateral information in support of an ongoing investigation of contaminants and their effects on osprey (*Pandion haliaetus*) reproduction (Henny unpub. data) (Fig. 1). Like those in the Mississippi basin sampled in 1995, the stations in the Columbia River and Rio Grande basins are affected by a wide range of agricultural, industrial, mining, and urban pollutants (Kelly 1996).

Organisms Selected For Sampling

The 1995 and 1997 projects represented a bridge between the NCBP and the BEST programs. Consequently, the fishes selected for sampling in the projects represented a blend of the historic NCBP fish sampling protocol and the guilds identified for monitoring aquatic habitats by the BEST program (BEST 1996). For the projects, the chosen guilds were sediment-dwelling or invertebrate-eating fish and piscivorous fish, these representing a combination of the recommended primary and secondary BEST program guilds (Appendix 1, Table A-2). The guild choices were necessitated by the need for rapid implementation of the projects and a corresponding lack of on-the-ground capacity for sampling piscivorous birds at the scale of the project.

Within the chosen guilds of fishes, preferred taxa were identified for three habitat classes — cold-, cool-, and warm-water — as they had been for the NCBP (Schmitt et al. 1990; Schmitt et al. 1999). For warm-water habitats, the preferred species were common carp (*Cyprinus carpio*) as the sediment-dwelling species and largemouth bass (*Micropterus salmoides*) or other black bass (i.e., *Micropterus* spp.) as the piscivore. These taxa were selected because they had historically been the ones most frequently collected at warm-water NCBP sites in the Mississippi basin (e.g., Schmitt et al. 1990; Schmitt et al. 1999), and their continued collection facilitates analyses of temporal trends, one of the stated objectives of the study and of the BEST program (BEST 1996; Schmitt et al. 1995). These are also species for which the chosen biomarkers have been validated, and there is consequently a literature against which to compare the findings of the studies.

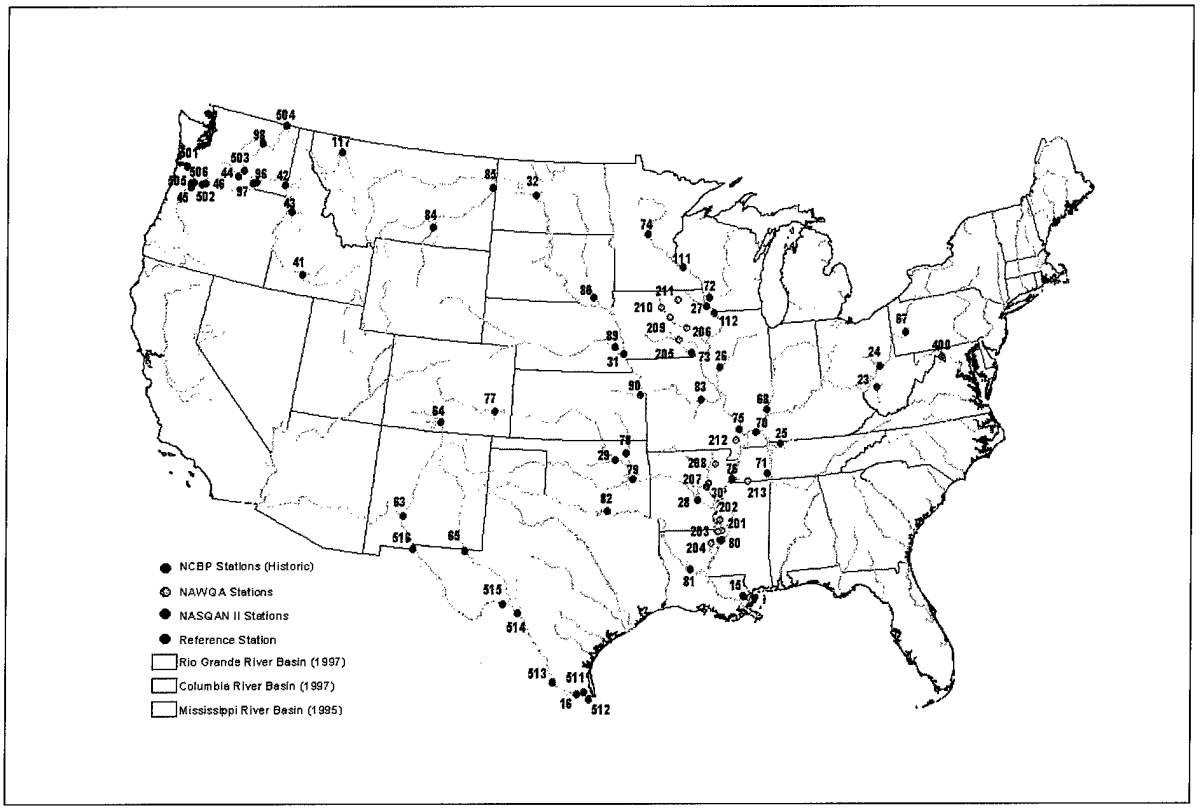


Figure 1. Stations sampled during the BEST projects in 1995 and 1997.

Within the chosen guilds and habitats (Appendix 1, Tables A-2 and A-3), alternate taxa were also identified for collection in the event that the preferred species could not be found. Alternative species for warm-water habitats were white bass (*Morone chrysops*) or other percichthyid (*Morone* spp.) for the piscivorous species and various suckers (Catostomidae) and catfishes (Ictaluridae) for the sediment-dwelling, invertebrate-eating species. An extensive body of literature exists for contaminant concentrations and biomarker performance in these taxa (e.g., Schmitt et al. 1990; Munkittrick et al. 1997; Schrank et al. 1997). In addition, suckers accumulate relatively high concentrations of a number of metals, including lead (Schmitt et al. 1985; Schmitt et al. 1993; Schmitt and Finger 1987). Alternative piscivores for cool-water habitats were the percids [walleye (*Stizostedion vitreum*) and sauger (*S. canadense*)] and northern pike (*Esox lucius*), which are all well-documented accumulators of mercury (Wiener and Spry 1996). In addition, risk of polyhalogenated hydrocarbons (PHHs) to wildlife has also been assessed using walleye (Giesy et al. 1995; Giesy et al. 1997). Rainbow trout (*Oncorhynchus mykiss*) and brown trout (*Salmo trutta*) were chosen as representative piscivores for cold-water habitats, and suckers were chosen as the sediment-dwelling taxa in both cool- and cold-water habitats. These taxa had also been collected historically at their respective NCBP sites, and the performance of many of the chosen biomarkers has been documented for them. In the Columbia River system, the northern pikeminnow (formerly northern squawfish, *Ptychocheilus oregonensis*) was permitted as an alternate piscivore; this species had been targeted in many previous NCBP collections and has been shown to accumulate mercury (Schmitt and Brumbaugh 1990).

Methods Evaluated

In the 1995 and 1997 projects, BEST program methods for aquatic habitats (BEST 1996) suited to fish were evaluated (Table 1). In addition to analyses of the fish carcasses for elemental and organic chemical residues (following NCBP protocol) and for stable isotopes of nitrogen ($\delta^{15}N$), methods included condition indices and gross and histopathological examination incorporating a quantitative necropsy-based field health assessment. Because of growing interest in the effects of chemicals on the endocrine system (e.g., Colborn 1991; Colborn et al. 1993; Guillette et al. 1994), biomarkers diagnostic of reproductive health and endocrine modulation or disruption in fish that were tested in an earlier study (Goodbred et al. 1994; Goodbred et al. 1997) were incorporated into the suite of fish health indicators for evaluation. These included plasma concentrations of reproductive steroid hormones (Guillette et al. 1994) and vitellogenin (Folmar et al. 1996). Furthermore, possible suppression of the immune system was investigated using two parameters indicative of immune system function — splenic macrophage aggregates (Blazer et al. 1994a; Blazer et al. 1997) and plasma lysozyme activity (Blazer et al. 1994a). These latter methods, ranging from organism-level to subcellular, were used as indicators of fish health. Health has been defined as the residual capacity to withstand stress. Hence, the more stressed (less healthy) an organism is, the less capable it is to withstand further stress (Bayne et al. 1985). Assessments of fish health attempt to integrate the overall responses of an organism to environmental stressors, including exposure to xenobiotics.

Two additional assays, the H4IIE rat hepatoma cell bioassay and hepatic ethoxyresorufin-*O*-deethylase (EROD) activity, were used together with analyses of fish carcasses for elemental and organic chemical residues to provide qualitative and quantitative information on the concentrations and biological effects of PHHs [e.g., polychlorinated biphenyls (PCBs), dioxins (PCDDs), furans (PCDFs) and polycyclic aromatic hydrocarbons (PAHs)]. The approach being evaluated assesses these complex groups of structurally and toxicologically similar contaminants without resorting to costly high-resolution analytical methods. In this approach, total PCB concentrations in whole fish were quantified along with organochlorine pesticides by gas chromatography with electron capture detection following NCBP protocol (Schmitt et al. 1990). Extracts from the composite samples were then screened for the presence of aryl hydrocarbon hydroxylase (AHH)-active compounds by the H4IIE bioassay (Tillitt et al. 1991), a sensitive *in vitro* method for documenting the cumulative concentrations of dioxin-like PHH residues. The assay was scaled to a 2,3,7,8-tetrachloro-*p*-dibenzodioxin (dioxin) standard and results were reported in units of total dioxin-equivalent concentrations. The H4IIE assay also responds to AHH-active PAHs (Willett et al. 1997), traces of which might occur in the whole-fish extracts if environmental PAH concentrations are extremely high (Baumann et al. 1982). To remove the PAHs and other labile compounds, the extracts were first subjected to a reactive cleanup procedure (Schwartz and Lehmann 1982), leaving only PHHs and other refractory compounds in the extracts to be assayed. Used in this manner, the H4IIE assay

Table 1. Methods incorporated into the large rivers projects.

Method	Description	Tissue(s) examined	Sensitivity	Primary reference(s)
Histopathology	Microscopic examination for the presence of lesions; can provide early indication of chemical exposure	Liver, gill, gonads, spleen, and kidney	Overall organism health and contaminants	Hinton et al. (1992); Hinton (1993); Goodbred et al. (1997)
Ethoxyresorufin-*O*-deethylase (EROD) activity	Enzyme induction by planar hydrocarbons	Liver	PCBs, PAHs, dioxins, and furans	Pohl and Fouts (1980); Kennedy and Jones (1994)
Lysozyme activity	A disease resistance factor that can be suppressed in the presence of contaminants	Blood plasma	Overall organism health	Blazer et al. (1994a)
Macrophage aggregate analysis	Macrophages are important in the immune system, serving as a first line of defense for the organism and as an antigen processing cell	Spleen, hemopoetic kidney, and liver	Multiple contaminants including PAHs and metals	Blazer et al. (1994a); Blazer et al.(1997)
H4IIE bioassay	A screening tool to determine the presence of certain classes of planar halogenated compounds	Whole fish (composites)	PCBs, dioxins, furans, and PAHs	Tillitt et al. (1991)
Vitellogenin	A precursor of egg yolk, normally synthesized in the liver of female fish	Blood plasma	Endocrine modulating compounds	Folmar et al. (1996)
Sex Steroids (estradiol and testosterone)	Determine reproductive health and status	Blood plasma	Endocrine modulating substances	Guillette et al. (1994); Goodbred et al. (1997)
Chemical analyses	Organochlorine chemical residues and elemental contaminants	Whole fish (composites)	Specific analytes	Schmitt et al. (1999)
Somatic indices	The relative mass of some organs is often indicative of chemical exposure	Gonads, spleen, liver	Overall organism health	Grady et al. (1992)
Stable N isotopes (^{14}N and ^{15}N)	The ratio of ^{15}N to ^{14}N ($\delta^{15}N$) increases with trophic position and sewage pollution	Whole fish (composites)	Trophic position, nitrogen sources	Cabana and Rassmussen (1996)
Necropsy-based fish health assessment	Visual assessment of external/internal anomalies (e.g., lesions, parasites, tumors), which may indicate contaminant-related stress	All	Overall organism health	Goede (1988, 1996); Adams (1990); Adams et al. (1993)

provides quantitative information on the cumulative concentration of AHH-active PCBs, chlorodioxins, chlorodibenzofurans, and related compounds of great concern, and thereby augments the information on total PCBs provided by the chemical analysis of the fish carcasses. To assess the cumulative exposure of the fish to all AHH-active compounds, including PAHs, the activity of the EROD enzyme in the livers of the individual fish was measured (Pohl and Fouts 1980). By comparing EROD results with those from the other two endpoints, the classes of chemicals responsible for the cumulative effect on EROD could be deduced (Table 2).

Table 2. Monitoring and assessment strategy for polycyclic aromatic and polyhalogenated hydrocarbons (PAHs and PHHs).

	Contaminants		
Endpoint	**PCBs**	**PCDDs & PCDFs**	**PAHs⁺**
GC-ECD[1] (carcass)	+	-	-
EROD[2] activity (liver)	*	*	*
H4IIE assay (carcass)[3]	*	*	-

[1] total PCBs by gas chromatography with electron-capture detection
[2] 7-ethoxyresorufin-*O*-deethylase
[3] after reactive cleanup to remove AhR-active PAHs
* AhR-active isomers and congeners only
⁺ and other planar organic compounds

The following reports summarize extant information on the biological markers and evaluate their value to the BEST program. More in-depth reviews and evaluations of these methods are being prepared as a corollary activity of the BEST program (e.g., Whyte et al. in prep.). Analytical methods for the measurement of elemental and organochlorine chemical concentrations in fish are well documented and are consequently not described in this summary.

EROD ACTIVITY

Jeff J. Whyte and Donald E. Tillitt

Measurement of ethoxyresorufin-*O*-deethylase (EROD) activity in fish is a well-established *in vivo* biomarker of exposure to certain planar halogenated and polycyclic aromatic hydrocarbons (PHHs and PAHs) and other structurally similar compounds (Bucheli and Fent 1995; Stegeman and Hahn 1994). EROD is a highly sensitive indicator of contaminant uptake in fish, providing evidence of receptor-mediated induction of cytochrome P450-dependant monooxygenases (the CYP1A subfamily specifically) by xenobiotic chemicals. Numerous laboratory experiments, simulated field studies, and natural field studies have examined EROD induction in more than 150 species of fish. In addition to PHHs and PAHs, an extensive list of individual contaminants and complex environmental mixtures has been examined for teleost EROD potential. Although EROD activity is best viewed as an indicator of exposure, the relationship between EROD and biological effects at higher levels of organization is the subject of intense investigation. It is becoming clear that the mechanism of CYP1A induction is closely related to, if not directly involved in, detrimental effects such as apoptosis and embryonic mortality seen in fish exposed to EROD-inducing contaminants (Cantrell et al. 1996). Apart from xenobiotic induction, EROD activity can be influenced by a large number of abiotic and biotic factors such as water temperature, age and reproductive phase (Andersson and Förlin 1992). An understanding of these factors is critical to the design and interpretation of field studies utilizing this biomarker.

Background

Over two decades have passed since the induction of the cytochrome P450 1A subfamily of monooxygenases (CYP1A) was proposed as a biomarker of exposure to PHHs and PAHs (Payne 1976; Payne and Penrose 1975). The foundations for this suggestion originated from the extensive work on this enzyme system performed in mammals dating back to the mid-1960s (Conney 1967; Mason et al. 1965). The cytochromes P450 are a diverse multigene family of heme-containing proteins that oxidize, hydrolyze, or reduce compounds through the insertion of an atom of atmospheric oxygen to the substrate during the reaction cycle (Nebert et al. 1993; Nelson et al. 1996). In

fish, these enzymes are concentrated mainly in the liver, but have been detected in the kidney, gastrointestinal tract and gill tissue (Varanasi 1989). Embedded in the smooth endoplasmic reticulum, they metabolize both endogenous and exogenous compounds (phase I reactions), generally increasing the water solubility of substrates, thereby enhancing their elimination (Andersson and Förlin 1992). In this way, cytochromes P450 such as CYP1A tend to detoxify xenobiotic chemicals; however, the phase I metabolites of some PAH and other contaminants may be more toxic than the parent compound (Guengerich and Liebler 1985).

The most useful aspect of CYP1A for biomonitoring purposes is the enzyme's tendency to increase in concentration upon chemical exposure. Induction of CYP1A is mediated through the binding of xenobiotics to a cytosolic aryl hydrocarbon receptor (AhR) (Fig. 2). AhR ligands generally have isoteric configurations and are similar in structure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (2,3,7,8-TCDD), a model CYP1A inducer (Fig. 3). Receptor binding is followed by a series of molecular events leading to the expression of several genes (including CYP1A) known as the "Ah-gene battery" (Nebert et al. 1993). The toxic effects of PHHs and structurally similar compounds are thought to be mediated through the AhR, with induced proteins causing alterations in cellular homeostasis (DeVito and Birnbaum 1994). In mammals, these effects include wasting syndrome, tumor promotion and thymic atrophy (Poland and Knutson 1982). In fish, early life stages appear to be particularly sensitive to AhR ligands (Mehrle et al. 1988; Walker and Peterson 1991), and recent evidence indicates the involvement of CYP1A enzymes specifically in this toxic response (Cantrell et al. 1996).

The use of CYP1A induction as an assessment technique has increased in recent years. This is due mainly to the optimization of protocols for the rapid and relatively inexpensive measurement of its catalytic activity as EROD (Kennedy and Jones 1994; Burke and Mayer 1974; Pohl and Fouts 1980). EROD induction as a biomarker in teleost species has several advantages. By indicating the induction of CYP1A, EROD activity provides a fingerprint of the presence of AhR-active compounds in fish. Historically, assessing the degree of uptake of these compounds was complicated by both their vast number and their varying degrees of bioavailability.

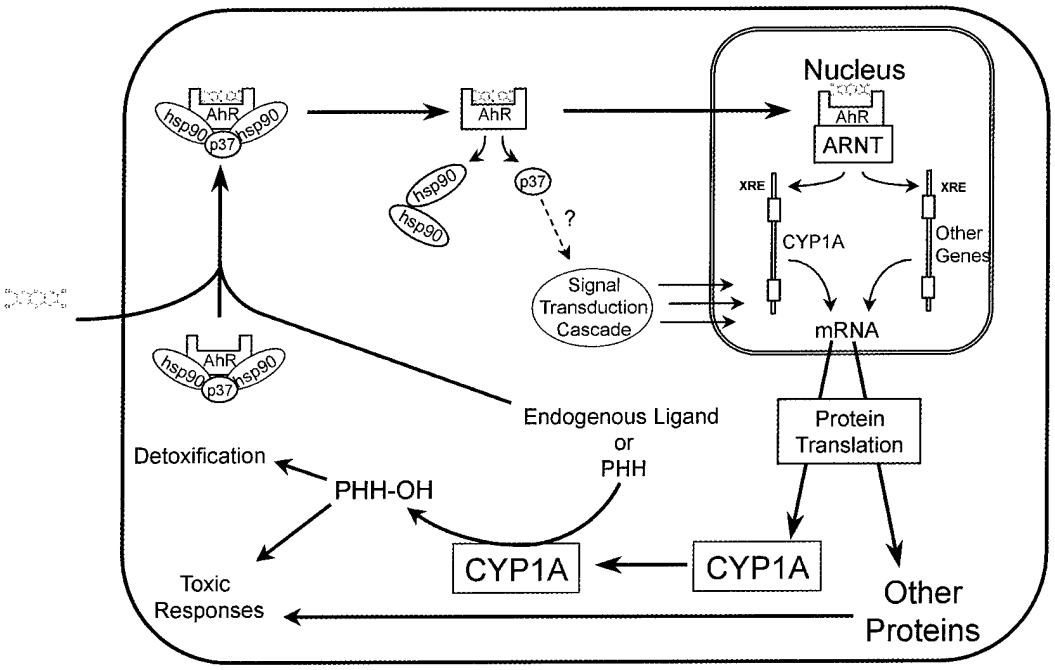


Figure 2. Proposed mechanism of AhR-mediated toxicity. Signal transduction by dioxin-like ligands is mediated by the AhR, which forms a transcription factor complex with an aryl hydrocarbon nuclear translocator protein (ARNT). This heterodimer recognizes specific DNA sequences, dioxin responsive elements (DRE), and leads to the induction of several genes (the Ah gene battery). The elevated levels of the protein products are thought to be involved in the toxic action of AhR ligands.

Although analytical measurements can provide the identities and concentrations of organic contaminants in fish tissues (Huestis et al. 1996; Firestone 1991), they do not render a direct indication of biological potency. Induction of EROD is an extremely sensitive indicator of environmental alterations and is usually one of the first detectable, quantifiable responses to exposure (Stegeman 1992). In addition, EROD represents the cumulative impact of all inducing chemicals, whether or not they are detected analytically.

Performing the EROD Assay

EROD activity describes the rate of the CYP1A-mediated deethylation of the substrate 7-ethoxyresorufin (7-ER) to form the product resorufin. The catalytic activity towards this substrate is an indication of the amount of enzyme present and is measured as the concentration of resorufin produced per mg protein per minute (mol/mg/min) (Kennedy and Jones 1994). Because metabolism is generally highest in hepatic tissue, the assay is typically conducted using fish liver. Determination of EROD activity involves two stages. The first stage is fish capture (typically 7-10 individuals per site) followed by the excision and cold preservation of the liver tissue. The excised tissue is homogenized and centrifuged to isolate fragments of the endoplasmic reticulum. This microsomal fraction contains the CYP1A enzymes of interest (Pohl and Fouts 1980). Consistency in terms of liver section removed, cryopreservation technique and microsomal preparation are important means of reducing individual sample variability (Pluta 1995; Heinonen et al. 1996). The second stage, the actual enzymatic assay, involves providing the microsomal fraction with 7-ER and NADPH and fluorometrically measuring resorufin production. Samples are then standardized based on protein content of the liver homogenate (Lorenzen and Kennedy 1993; Lowry et al. 1951; Bradford 1976).

Factors That Can Affect EROD Induction in Fish

As with most biological phenomena, EROD in the tissues of an organism is influenced by a variety of internal, external, and temporal factors (Bucheli and Fent 1995). Biological factors that can influence EROD activity include species (Addison et al. 1991; Segner et al. 1995), fish size and age (Peters and Livingstone 1995; Pluta 1993), and reproductive status (Campbell et al. 1976; Schreck and Hopwood 1974). The physical treatment of fish in both laboratory and field studies can also greatly affect EROD measurements. Careful consideration should be given to contaminant exposure route (James and Bend 1980; Haasch et al. 1993), fish diet (Jimenez and Burtis 1988), and the use of anesthesia during capture (Kleinow et al. 1986). Environmental variables such as temperature and pH can drastically affect induction of EROD and should be routinely measured throughout a study (Andersson and Koivusaari 1985; Sleiderink et al. 1995; Willis et al. 1991). Contaminant exposure period and study duration are important in both laboratory and caged-fish studies

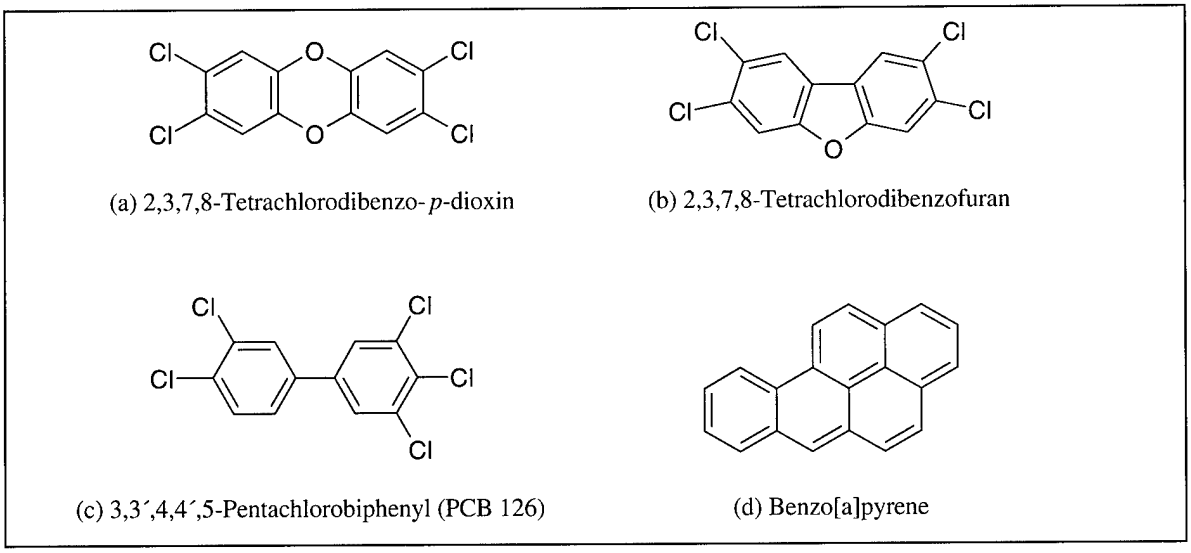


Figure 3. Representative AhR ligands. The molecules demonstrate the general structure of compounds in the following classes: (a) polychlorinated dibenzo-p-dioxins (PCDDs), (b) polychlorinated dibenzofurans (PCDFs), (c) polychlorinated biphenyls (PCBs) and (d) polycyclic aromatic hydrocarbons (PAHs).

examining EROD (van der Weiden et al. 1994b; Sleiderink and Boon 1996).

A variety of chemicals and chemical mixtures are known to inhibit the induction of EROD in fish. These include organic, organometallic, and metallic compounds such as specific polychlorinated biphenyl (PCB) congeners (Gooch et al. 1989; Newsted et al. 1995), and organotins (Bucheli and Fent 1995). In addition to antagonistic chemicals, there a variety of AhR agonists that are of biogenic nature, including plant metabolites and biotoxins (Takahashi et al. 1995). In short, the presence or absence of EROD activity in fish from a site may not always represent contamination by or lack of traditional AhR agonists, and study designs incorporating a battery of tests for biological responses to contaminants will likely yield more concrete information.

Value and Utility of EROD in the BEST Program

As a monitoring tool, EROD activity provides a relatively rapid indication of toxic planar compound uptake in fish. For this reason, EROD is often termed an "early warning system" (Payne et al. 1987). Thus far, hundreds of field studies have employed the EROD assay to determine spatial and temporal trends of contamination in aquatic systems (Kennedy and Jones 1994; Balk et al. 1993; Teal et al. 1992; Adams et al. 1996; Achazi and Leydecker 1992). These studies have detected EROD induction in many species of fish from a broad range of habitat types. This extensive validation of EROD as a biomarker has led to its use in several contaminant monitoring programs worldwide [e.g., North Sea Task Force, (Stagg 1991; Pluta 1995), Environment/Cellulose (Förlin et al. 1995), Mediterranean Pollution Network (Burgeot et al. 1996), French National Observation Network (Godefroy et al. 1996)].

Ideally, a biomarker will exhibit a relationship to toxicity in the organism(s) being examined. Induction of CYP1A (estimated from EROD activity), while not a toxic response per se, does indicate the potential for AhR ligands to induce biochemical change. The generation of reactive PAH intermediates by CYP1A has long been known as a source of DNA adducts that can lead to carcinogenesis [Fig. 4,

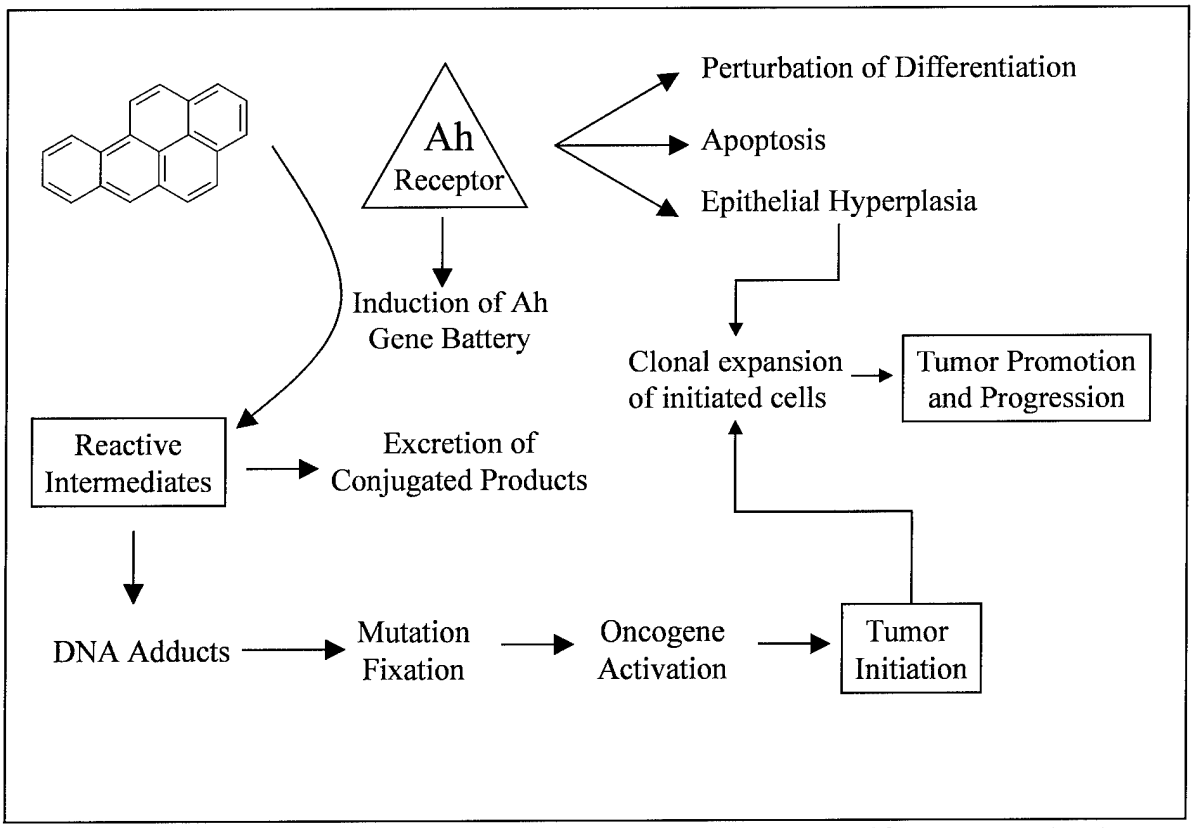


Figure 4. Ah receptor-mediated formation of DNA adducts and tumor initation upon exposure to benzo[*a*]pyrene. Compounds such as 2,3,7,8-TCDD are also capable of perturbing differentation, affecting apoptotic balance and causing tissue-specific proliferation, another hypothesized route of AhR-mediated carcinogensis (From Nebert et al. 1993; reproduced by permission).

(Varanasi et al. 1989)]. In addition, a variety of parameters in fish have been associated with the induction of EROD activity including reproductive effects (e.g. reduced serum steroid levels), increased liver somatic index and mortality (Watson and Di Giulio 1997; van der Weiden et al. 1994a; Levine et al. 1995; Gagnon et al. 1994). At present the many symptoms and effects that AhR ligands exert on a wide variety of biochemical systems have made it difficult to identify the critical events that lead to toxicity. Until a more direct linkage between CYP1A induction and detrimental effects in fish is established, EROD activity is best viewed as an indicator of contaminant exposure rather than of effect. As more information is gathered on the relationship between EROD activity and detrimental effects in fish, this biomarker may also serve as a predictive tool for contaminant risk assessment. In the meantime, EROD induction serves as an economical assessment tool for environmental managers, allowing them to prioritize areas for examination using more expensive techniques such as congener specific analysis of tissue and sediment.

By employing the teleost EROD induction technique with other methods (e.g. H4IIE bioassay, instrumental analysis), an integrated assessment of the contaminant levels and identities in fish will be provided. This multilevel approach renders the best balance of information at the minimal cost.

H4IIE BIOASSAY

Jeff J. Whyte and Donald E. Tillitt

The H4IIE rat hepatoma cell line bioassay (H4IIE bioassay) is an *in vitro* test used to detect and semiquantify specific contaminants and classes of contaminants chemically extracted from environmental matrices such as sediment, water, and organisms (whole or specific tissues). The H4IIE bioassay measures the catalytic activity of cytochrome P4501A (CYP1A), a mixed-function oxidase (MFO) enzyme, as 7-ethoxyresorufin-*O*-deethylase (EROD) activity in cultured rat liver cells exposed to environmental extracts. EROD is induced by, and the H4IIE bioassay is consequently useful for characterizing, the presence of certain polycyclic aromatic hydrocarbons (PAH) and related compounds (e.g., nitrogen heterocyclics and sulfur-, oxygen-, nitro-, amino,- and alkyl-substituted PAH) and polyhalogenated hydrocarbons (PHH) in environmental samples. The PHHs include the highly toxic and persistent polychlorinated dioxins (PCDDs), dibenzofurans (PCDFs), biphenyls (PCBs), and naphthalenes, as well as the brominated analogs of these compounds. These classes of compounds induce CYP1A and hence EROD activity in cells by binding to the cytosolic aryl hydrocarbon receptor (AhR). This AhR-mediated mechanism of EROD induction is believed to be involved in many of the toxic effects associated with PHHs and PAHs (Poland and Knutson 1982) (Fig. 3). An overview of EROD induction is presented in "EROD Activity."

The H4IIE bioassay has advantages over traditional analytical chemistry techniques in that it reveals the cumulative biological activity of numerous structurally similar contaminants, each with differing potencies. This assay can also reveal the potential interactions that can occur between contaminants when they are present in environmental samples as complex mixtures. The H4IIE bioassay is valuable for environmental monitoring purposes because it enables the assessment and ranking of the potential toxicity of samples based on their ability to induce EROD as a surrogate for analytical determination of specific compounds. When based on tissue samples, cumulative H4IIE-derived potency estimates can be used to assess the risk to the organism(s) from which the extract was obtained. Such estimates can also be used to estimate the contaminant burden or dose that the organism could contribute either to higher trophic levels (via the food chain) or to its progeny (via maternal transfer). The H4IIE bioassay has a high degree of sensitivity (detection limit < 10 femtomoles 2,3,7,8- tetrachlorodibenzo-*p*-dioxin [TCDD]) and can be rapidly performed. These characteristics make the H4IIE bioassay an ideal tool for evaluating the toxic potential of the samples collected for monitoring programs.

Background

The continuous cell line, H4IIE, was derived from the Reuber Hepatoma H-35 (Reuber 1961) by Pitot and coworkers (Pitot et al. 1964). A decade later, induction of CYP1A catalytic activity in the cell line was demonstrated (Benedict et al. 1973; Niwa et al. 1975). The H4IIE cells are well-suited for the examination of EROD induction due to their excellent growth characteristics and the presence of low basal, but highly inducible, CYP1A activity. The H4IIE cell line is exquisitely responsive to 2,3,7,8-TCDD-based CYP1A induction. These characteristics prompted researchers from the U.S. Food and Drug Administration to develop and characterize a contaminant detection bioassay based on the H4IIE cell line (Bradlaw and Casterline 1979). This original assay

was used to screen for the presence of CYP1A-inducing chemicals in foodstuffs as indicated by aryl hydrocarbon hydroxylase (AHH) activity, a catalytic measure of CYP1A (Trotter et al. 1982; Casterline et al. 1983). The assay was subsequently modified to examine EROD activity rather than AHH activity (Sawyer and Safe 1982) because the EROD assay employs a non-toxic substrate.

Use of the H4IIE assay to rank the toxic potency of individual chemicals based on the 2,3,7,8-TCDD equivalency (TEQ) concept was first proposed by Safe (1987). The TEQ concept was subsequently expanded and used to demonstrate the cumulative toxicity of mixtures of PAHs and PHHs in controlled laboratory studies and in extracts of environmental samples. TEQ values generated by the H4IIE assay provided a relative toxicity estimate for individual chemicals. The values can also be used together with analytical chemistry to evaluate the potential interactions of mixtures of CYP1A-inducing chemicals in biological systems. More recently, environmental assessments using the H4IIE bioassay have become more prevalent, mainly due to the systematic characterization of the assay by Tillitt et al. (1991). Additional modifications that have improved the bioassay (e.g., microtiter plates, use of live cells) have also been introduced over the past decades (Tysklind et al. 1994; Whyte et al. 1998; Bradlaw et al. 1982; Donato et al. 1992; Munkittrick et al. 1993).

Performing the H4IIE Bioassay

Conduct of the H4IIE bioassay can be divided into three general stages (Fig. 5): 1) Extraction, cleanup, fractionation, and analytical characterization of contaminants in the tissue or other environmental matrix; 2) preparation of an extract dilution series with which the cells are dosed; and, 3) measurement of EROD activity and calculation of TEQs (expressed as pg 2,3,7,8-TCDD/g tissue). Extraction, cleanup, and fractionation methods depend on the contaminant classes of interest, but generally involve isolation of hydrophobic planar compounds such as PHHs (Feltz et al. 1995; Huestis et al. 1995). Whole extracts can be examined for general inducing potency. Alternatively, fractionated extracts can provide more detailed information on the contribution of different classes of contaminants (e.g., PCBs, PCDDs/Fs) (Gale et al. in press). Analytical detection techniques such as GC/MS or HPLC can identify specific chemicals in extracts potentially responsible for EROD induction in H4IIE cells, but the cost of this analysis is avoided if the bioassay is being used simply to indicate and quantify the cumulative presence of EROD-inducing compounds in extracts.

Prior to dosing the H4IIE cells, isolated contaminants are transferred into a solvent carrier suitable for delivery to the cultured cells (e.g., isooctane or DMSO) and a logarithmic dilution series is prepared (Tillitt et al. 1991; Smith et al. 1994). H4IIE cells, cultured under conditions described by Tillitt et al. (1991), are seeded in microtiter plates and grown for 24 h. Cells are dosed with extracts or a 2,3,7,8-TCDD standard, incubated (24 – 72 h) and assayed for EROD using a modified method of Pohl and Fouts (1980). The microtiter plate method allows for measurement of both EROD and protein in the same wells (Kennedy and Jones 1994). At the final stage of the assay, concentration-response curves are used to determine relative extract potencies (TEQs) by comparing EC50 or slope values of the extract to those of 2,3,7,8-TCDD (Mason et al. 1985; Tillitt et al. 1993).

Factors That Can Affect the H4IIE Bioassay

Because the H4IIE bioassay is a laboratory assay, many of the external modifying factors that can influence *in vivo* measurement of EROD in fish (e.g., sex, species, ambient temperature) do not influence EROD induction in H4IIE. Therefore, deviations from specified experimental conditions are the most likely source of variability in the H4IIE bioassay. Similar to the *in vivo* assay, variables such as reagent temperature and pH, and resorufin and ethoxyresorufin purity may influence EROD measurements. Other factors specific to cell culture (e.g., cell passage number, mycoplasma contamination) may also influence EROD measurements in H4IIE.

The assessment of environmental extracts with the H4IIE bioassay may also be affected by the presence of specific compounds in the mixture (e.g., certain PCBs). This is mainly caused by inhibition of the catalytic activity of CYP1A in H4IIE and can lead to erroneously reduced EROD measurements (Sawyer et al. 1984). An elegant solution to this problem was presented by Denison and coworkers (Garrison et al. 1996), who transfected a luciferase gene that is expressed upon AhR-complex binding to DNA in H4IIE cells. This results in induction of luciferase activity, which produces luminescence, upon exposure to CYP1A-inducing compounds. The luciferase activity is not influenced by substrate inhibition, resulting in enhanced sensitivity to AhR ligands and greater confidence in induction measurements. It is yet to be determined whether this modified technique will replace the traditional H4IIE bioassay.

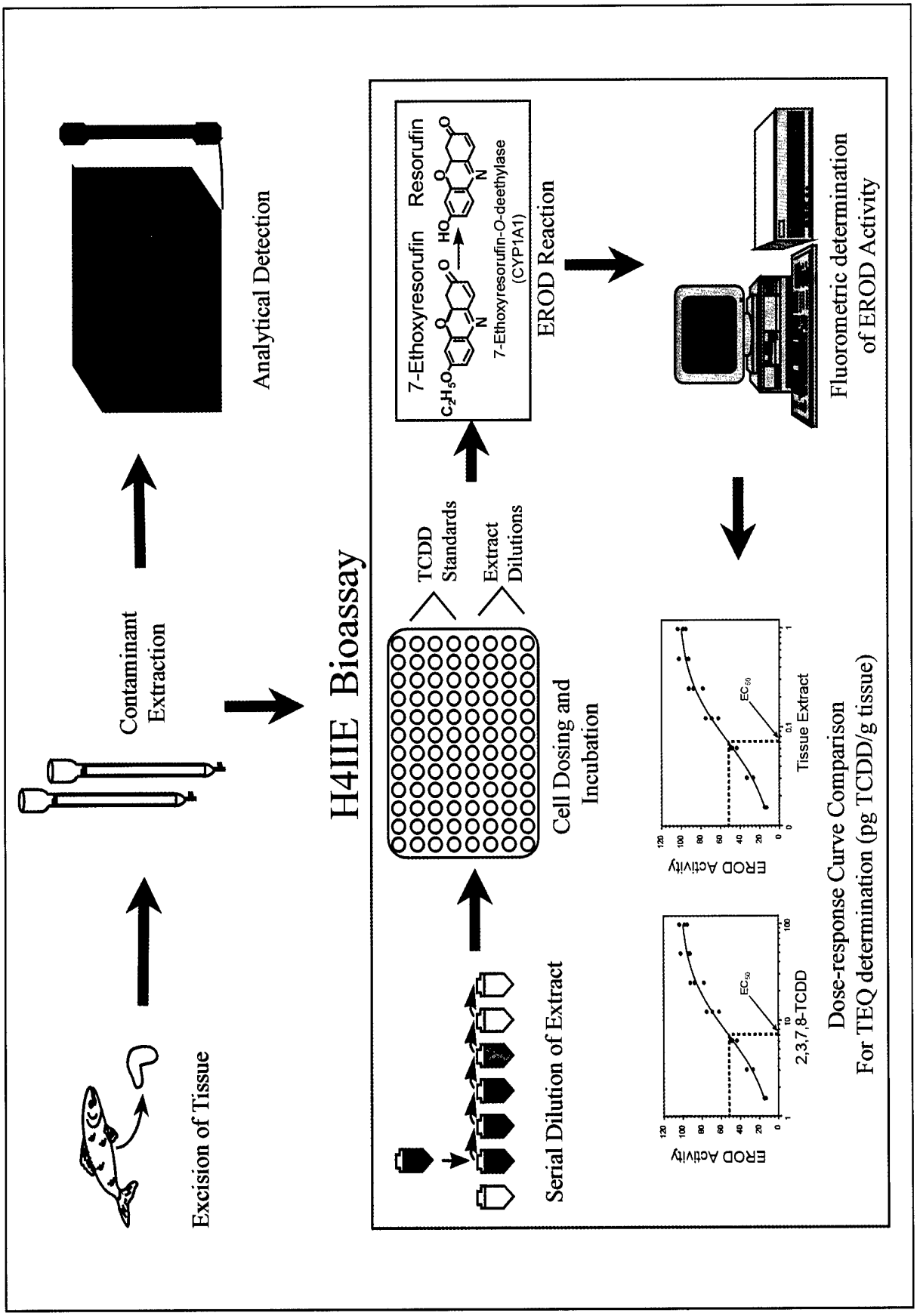


Figure 5. Application of the H4IIE cell line bioassay to assess the toxic potency of environmental extracts. Extract EROD-inducing potency in the cells is compared to that of 2,3,7,8-TCDD to generate a TCDD equivalent (TEQ) concentration (pg TCDD/g tissue).

Value and Utility of the H4IIE Bioassay in the BEST Program

The persistence and demonstrated toxic effects of PHHs are of great concern to the U.S. Fish and Wildlife Service and other natural resource management agencies (U.S. Fish and Wildlife Service 1993). The H4IIE bioassay is a rapid and highly sensitive test that can indicate the presence of PHHs and related compounds in environmental samples and in some situations can estimate the potential cumulative toxicity of these compounds to organisms. The bioassay can also provide information on the biological interactions of PHHs when used in concert with analytical chemistry methods. The H4IIE bioassay is also of value because of its ability to relate the EROD-inducing strength of PHHs to potential toxic impacts in whole organisms. Relationships between CYP1A enzymatic activities in H4IIE cells and deleterious effects in live rats have been demonstrated for individual PCDD (Bradlaw et al. 1980), PCDF (Mason et al. 1985; Bandiera et al. 1984), and PCB (Sawyer and Safe 1982; Leece et al. 1985) congeners. The strong correlation observed between AHH induction by these congeners in cultured cells and weight loss or thymic atrophy in whole organisms supports the use of the *in vitro* bioassay as a tool for predicting toxic potency (reviewed by Safe 1990).

Complex mixtures of PHHs have also been examined using the H4IIE bioassay and have been used to assess toxic impacts in wildlife. For example, hatching success of double-crested cormorants (*Phalacrocorax auritis*) in Great Lakes colonies was strongly correlated with TEQ results from the H4IIE bioassay (Fig. 6), whereas conventional contaminant analysis correlated poorly with hatching success of eggs from the same colony (Tillitt et al. 1992). Mammalian studies have also revealed the utility of the H4IIE bioassay to predict toxic effects. Mink (*Mustela vison*) fed a diet containing increasing percentages of fish from a contaminated bay in the Great Lakes showed reproductive effects that were related to results from the H4IIE bioassay (Tillitt et al. 1996). By taking into account the differing biological potencies of the chemical components in an extract, the H4IIE bioassay is a good predictor of the toxic outcome of exposure to the extract.

The H4IIE bioassay is among the Tier I or "workhorse methods" recommended for screening environmental samples for dioxin-like activity in virtually all habitats and guilds likely to be sampled by the BEST program (BEST 1996). The assay can detect extremely low concentrations of contaminants

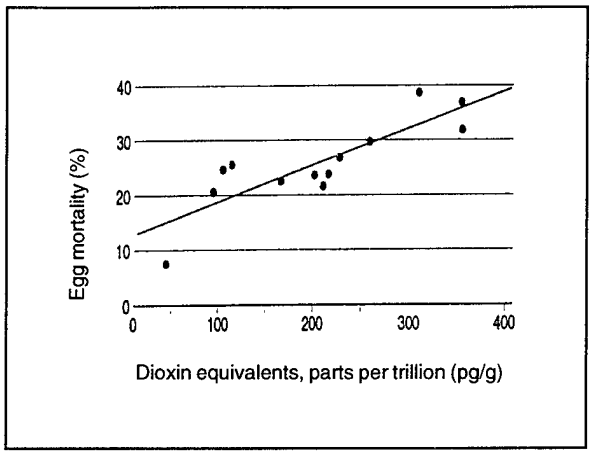


Figure 6. Hatching success in Great Lakes colonies of double-crested cormorants versus PCBs in eggs as dioxin-equivalent concentrations, 1987-1988 (modified and redrawn from Tillitt et al. 1992 by permission); each point represents the mean for a colony.

that are a high priority for the BEST program (PHHs and PAHs; U.S. Fish and Wildlife Service 1993). Used in conjunction with other methods (e.g., *in vivo* measurement of hepatic EROD and instrumental analysis of total PCB residues in the carcasses of the fish), the H4IIE bioassay can provide information on the class or classes of contaminants in the samples inducing the MFO system without the expense of high-resolution chemical analyses. It should be noted that the *in vivo* and *in vitro* measures of EROD activity provide similar but distinctly different information. Hepatic EROD in fish indicates the presence of compounds that have already interacted with the AhR, whereas the H4IIE bioassay reveals contaminants accumulated in tissues that have the potential to bind to the AhR. Use of these two assays in concert and with specific fractionation schemes can yield critical information regarding the presence of CYP1A inducers that are easily metabolized or otherwise non-persistent. Perhaps most importantly, and in contrast to many biomarkers, TEQs in biological samples as estimated by the H4IIE bioassay indicate the presence of compounds that are known to exert toxic effects through a similar mode of action. Although questions have been raised about species-specific differences in response to PHH and PAH congeners (Clemons et al. 1997), extant information suggests that the mechanism of AhR-mediated CYP1A induction is similar among vertebrates (Stegeman and Hahn 1994), supporting the use of H4IIE in the assessment of toxic potency of contaminants accumulated in organisms such as fish. These attributes give the H4IIE bioassay predictive power in terms of risk to organisms.

CONDITION FACTOR AND ORGANO-SOMATIC INDICES

Gail M. Dethloff and Christopher J. Schmitt

Measurements of condition factor, which relates weight to length, and organo-somatic indices, which indicate the proportional sizes of certain organs, are standard procedures in fish physiology studies and in fisheries biology. The condition factor is an organism-level response, with factors such as nutritional status, pathogen effects, and toxic chemical exposure causing greater-than-normal or less-than-normal weights. Organo-somatic indices reflect the status of organ systems, which may change in size due to environmental factors more rapidly than organism weights and lengths increase or decrease. Both the condition factor and organo-somatic indices are used as indicators of the well-being of individual organisms.

Background

Condition factor and organo-somatic indices have been used extensively in fish health and population assessments (reviewed by Goede and Barton 1990; Hoque et al. 1998). Because it integrates many levels of sub-organismal processes (e.g., molecular, cellular, organ system), an index such as Fulton's condition factor (Carlander 1969) may signify the overall condition and nutritional status of individual fish (Adams et al. 1992a). The size or weight of the liver, spleen, and gonads relative to fish length or weight may also signify overall health and reproductive status. The organo-somatic indices are generally expressed as percentages of total body weight. The hepato-somatic index (HSI) is the weight of the liver expressed as a percentage of total body weight; it is also known as the liver somatic index. Gingerich (1982), in summarizing the extensive literature on the biology of the fish liver, reported that the liver constitutes, on average, about 2% of body weight in mature teleost fishes. Alterations in liver size may reflect changes in the metabolism and energy reserves of an individual fish (Busacker et al. 1990). The spleno-somatic index (SSI) is the weight of the spleen expressed as a percentage of total body weight. Alterations in this index could indicate an abnormal condition in the spleen such as necrosis or swelling due to infection (Goede and Barton 1990). The gonado-somatic index, the weight of the gonads expressed as a percentage of total body weight, is discussed in "Reproductive Indicators."

Measuring Condition Factor and Organo-somatic Indices

Measurements of condition factor and organo-somatic indices require only measuring boards and balances of the appropriate capacity to measure length of whole fish and to weigh whole fish and organs. To avoid moisture losses and gains and corresponding weight changes during storage (i.e., freezing or preservation), weight determinations should be made on live or freshly killed specimens (Busacker et al. 1990). Condition factor is generally computed as weight/length^3. This equation reflects the expected exponential gain in weight relative to length as fish grow. Depending on the units of measurement, a constant may be included to bring the index values near unity (Anderson and Gutreuter 1992). Relationships between length and weight also may be estimated by regression analysis (length-weight equations) (Carlander 1969). Organo-somatic indices are often calculated as (organ weight/body weight) * 100 (Busacker et al. 1990). Alternatively, these relationships may be documented statistically (i.e., as regression coefficients of the relationships) (Delahunty and de Vlaming 1980; Grady et al. 1992). If ratios are calculated, they can be problematic because they do not conform to any common statistical distributions. An alternate approach to the appraisal of relative organ size and weight relative to length is through a procedure such as the analysis of covariance, whereby the nature of the relations between the variables is examined (Delahunty and de Vlaming 1980; Grady et al. 1992). However, the requirement of more-or-less equal-size fish to meet the assumptions for analysis of covariance may preclude this option for some comparisons.

Factors That Can Affect Condition Factor and Organo-somatic Indices

In general, condition factor varies directly with nutrition (Tyler and Dunn 1976). A negative correlation has been seen between disease and condition in fishes (Möller 1985). Condition factor may vary in either direction outside the normal range in response to chemical exposure. Elevated condition factors have been found in white sucker (*Catostomus commersoni*) and redbreast sunfish (*Lepomis auritus*) at sites polluted with pulp mill effluents (Adams et al. 1992a; McMaster et al. 1991). Decreased condition factors have been seen in white sucker at sites with elevated concentrations of metal mixtures and in Atlantic cod

(*Gadus morhua*) exposed to petroleum (Munkittrick and Dixon 1988; Miller et al. 1992; Kiceniuk and Khan 1987). Nutrition, disease and contaminants are highly inter-related in terms of their effects on fish condition. Insufficient nutrition can lead to higher disease susceptibility (Klontz 1985) and thus altered condition factor. There is evidence that contaminants may potentiate disease outbreaks (Sindermann 1990; Möller 1985) and that they can alter food resources (Munkittrick and Dixon 1988). Incidence of disease or poor food resources can then manifest as lowered condition factor.

Condition factor may also vary seasonally (Griffiths and Kirkwood 1995; Saborowski and Buchholz 1996), possibly due to changes in food availability or metabolism, and with changes in gonadal status (Chellappa et al. 1995). As one might expect, condition factor varies greatly among fish taxa owing to their differential architecture, but condition indices can also vary from location to location within a species (Doyon et al. 1988; Fisher et al. 1996). A final matter to consider when using condition factor to assess fish health is that a decrease in weight due to loss of energy stores can be offset by an increase in body water (Goede and Barton 1990).

Because of the energy storage and metabolic functions of the liver, alterations in liver size due to environmental stressors are of interest. Evaluation of the HSI must consider the role of both endogenous and exogenous factors. The HSI varies with seasonal cycles (Saborowski and Buchholz 1996; Delahunty and de Vlaming 1980; Beamish et al. 1996; Slooff et al. 1983). Because of the liver's role in storage and metabolism, nutritional quality and regimes also affect relative liver size (Daniels and Robinson 1986; Foster et al. 1993; Heidinger and Crawford 1977; Swallow and Fleming 1969). The HSI can also vary with sex and changes in gonadal status (Grady et al. 1992; Fabacher and Baumann 1985; Förlin and Haux 1990). In females, the HSI may change as the gonado-somatic index changes due to the liver's role in vitellogenesis (Scott and Pankhurst 1992).

Like the condition factor, the HSI is constrained by the allometry of the population, the species, or both (Grady et al. 1992). The form of the allometric relation between liver weight and body weight varies widely among species, both positively and negatively (Grady et al. 1992; Yakoleva et al. 1976). The fish liver may also store blood during periods of quiescence (Gingerich 1982), which suggests that the activity of the fish immediately prior to capture and the protocol used to procure the liver may affect relative liver size. Factors that cause a disproportionate change in body weight will also affect the HSI.

Of the organo-somatic indices, the HSI is the one most often associated with contaminant exposure (Adams and McLean 1985). Several investigators have suggested that relative liver enlargement in fish indicates exposure to environmental carcinogens or other toxic chemicals (Table 3). Increased HSI has been reported in brown bullheads (*Ameiurus nebulosus*) from sites polluted with polycyclic aromatic hydrocarbons (PAHs) (Fabacher and Baumann 1985; Gallagher and Di Giulio 1989), in rainbow trout (*Oncorhynchus mykiss*), Atlantic cod, and winter flounder (*Pleuronectes americanus*) exposed to waters containing a mixture of PAHs and other pollutants (Poels et al. 1980; Kiceniuk and Khan 1987; Fletcher et al. 1982), and in redbreast sunfish exposed to industrial discharge containing PAHs and polychlorinated biphenyls (PCBs) (Adams et al. 1989). Additionally, striped bass (*Morone saxatilis*) exposed to Hudson River water contaminated with PCBs, hard-head catfish (*Arius felis*) from PAH-contaminated sites, and European plaice (*Pleuronectes platessa*) collected from sites contaminated with sewage sludge all had enlarged livers (Buckley et al. 1985; Everaarts et al. 1993; Secombes et al. 1995). Fabacher and Baumann (1985) and Gallagher and Di Guilio (1989) concluded that increased xenobiotic metabolism in fish was achieved by enlarging the liver (and therefore increasing the HSI) rather than by increasing the specific activity of the detoxification enzymes.

In contrast with the studies described above, a number of laboratory studies found that liver size decreased following exposure to contaminants (Table 3). Exposure of rainbow trout to sodium pentachlorophenate (Hickie and Dixon 1987) caused a reduction in the HSI, as did exposure of perch (*Perca fluviatilis*) to a mixture of metals (Larsson et al. 1984); Atlantic salmon (*Salmo salar*) to cyanide (Ruby et al. 1987); Asian redtail catfish (*Mystus nemurus*) to hydrogen sulfide (Hoque et al. 1998); and striped mullet (*Mugil cephalus*) to crude oil (Chambers 1979). These decreases may have reflected glycogen loss in the liver as energy stores were utilized (Barton et al. 1987). Exposure to carbofuran also decreased HSI in the green snakehead (*Channa punctatus*); the decrease was linked to histopathological changes in the liver, including hepatocyte damage and degeneration (Ram and Singh 1988). Field studies investigating bleached kraft mill effluent (BKME) effects have found significantly lower HSI in exposed fish, though seasonal cycles may also have contributed to the decrease in liver size (Adams et al. 1992a; McMaster et al. 1991). Adams et al. (1992a) attributed lower HSI to altered carbohydrate metabo-

Table 3. Investigations in which the hepatosomatic index (HSI) has been evaluated as a biomarker of contaminant exposure.

Species	TSN[1]	Location	Contaminant(s)	Result	Reference
Field Studies					
Brown bullhead (*Ameiurus nebulosus*)	164043	Black River, Ohio	PAH	Increase	Fabacher and Baumann 1985
		North Carolina	PAH	Increase	Gallagher and Di Giulio 1989
Hardhead catfish (*Arius felis*)	164165	Gulf of Mexico	PAHs, cadmium	Increase	Everaarts et al. 1993
Bream (*Abramis brama*)	163666	European Rivers	Multiple	Increase	Slooff et al. 1983
Rainbow trout (*Oncorhynchuss mykiss*)	161989	Rhine River	Multiple	Increase	Poels et al. 1980
		Finland	Paper mill effluent	No effect	Oikari and Niittylä 1985
White sucker (*Castostomus commersoni*)	163895	Canada	Bleached kraft mill effluent	Decrease	McMaster et al. 1991
Striped bass (*Morone saxatilis*)	167680	Hudson River	Multiple (PCBs)	Increase	Buckley et al. 1985
Redbreast sunfish (*Lepomis auritus*)	168131	East Tennessee	PCBs, PAHs	Increase	Adams et al. 1992a
		East Tennessee	Bleached kraft mill effluent	Decrease	Adams et al. 1992a
European plaice (*Pleuronectes platessa*)	172902	Scotland	Sewage sludge	Increase	Secombes et al. 1995
Laboratory studies					
Rainbow trout (*Oncorhynchuss mykiss*)	161989	NA	Acidity	Decrease	Lee et al. 1983
		NA	Sodium pentachlorophenate	Decrease	Hickie and Dixon 1987
Atlantic salmon (*Salmo salar*)	161966	NA	Cyanide	Decrease	Ruby et al. 1987
Perch (*Perca fluviatilis*)	168470	NA	Metals (mixture)	Decrease	Larsson et al. 1984
Atlantic cod (*Gadus morhua*)	164712	NA	Crude oil	Increase	Kiceniuk and Khan 1987
Winter flounder (*Pleuronectes americanus*)	172904	NA	Crude oil	Increase	Fletcher et al. 1982
Brown bullhead (*Ameiurus nebulosus*)	164043	NA	Benzo(*a*)pyrene	No effect	Grady et al. 1992
Asian redtail catfish (*Mystus nemurus*)		NA	Hydrogen sulfide	Decrease	Hoque et al. 1998
Striped bass (*Morone saxatilis*)	167680	NA	Arsenic	Mixed	Blazer et al. 1997
Striped mullet (*Mugil cephalus*)	170335	NA	Crude oil	Decrease	Chambers 1979
Green snakehead (*Channa punctatus*)	166666	NA	Carbofuran	Decrease	Ram and Singh 1988

[1]Taxonomic serial number from the Interagency/International Taxonomic Information System (ITIS) database

lism.

Spleen size is considered a useful diagnostic factor because the spleen is a hematopoietic organ (Anderson 1990) and dysfunction could have effects at the whole-organism level. The SSI has not been as thoroughly investigated as the HSI, but certain endogenous and exogenous factors are known to affect it. The range of spleen sizes varies among fishes (Anderson et al. 1982; Ruklov 1979) and among populations of the same species (Lipskaya and Salekhova 1980; Ruklov 1979). Relative spleen weight may also differ with gender, age, size, gonadal development, and growth rate (Krykhtin 1976; Ruklov 1979). Seasonal changes also affect the SSI (White and Fletcher 1985). Finally, as with the HSI, factors that cause a disproportionate change in body weight will affect the SSI.

Nonspecific stressors (e.g., hypoxia) can result in altered spleen morphology. Studies on six species of teleost fish found that transient hypoxic conditions or severe exercise caused the spleen to contract fully and then decrease in size and hemoglobin content (Yamamoto and Itazawa 1985; Yamamoto 1988). Acute stressors including increased temperature, exhaustive exercise, hypoxia, and simulated transport led to spleen contraction and decreased spleen mass in young European sea bass (*Dicentrarchus labrax*), dab (*Limanda limanda*), and in *Trematomus bernachii,* a benthic Antarctic teleost (Hadj-Kacem et al. 1987; Davison et al. 1994; Pulsford et al. 1994). Alterations in spleen morphology due to nonspecific stressors are paralleled by alterations at the cellular level: the release of erythrocytes into circulation, a decrease in the total numbers of intact white blood cells, an increase in the proportion of atypical cells (erythrocytes and macrophage-like cells), and enhanced red blood cell degradation in the spleen (Yamamoto and Itazawa 1983; Maule and Schreck 1990; Peters and Schwarzer 1985). Histological changes (edema, necrosis, hyperaemia) were seen in the spleens of channel catfish (*Ictalurus punctatus*) held under conditions of sublethal hypoxia for 24, 48, and 72 h (Scott and Rogers 1980). Enlargement or swelling of the spleen, on the other hand, is considered to be indicative of disease or immune system problems (Goede and Barton 1990). This condition may be due to the increased hypertrophy or proliferation of leukocytes (Anderson 1990).

Chemical contaminants can also alter the SSI. A trend of elevated SSIs was seen with chronic BKME exposure of redbreast sunfish (Adams et al. 1992a). Decreased SSIs occurred in cunners (*Tautogolabrus adspersus*) exposed to petroleum for six months, in Atlantic cod exposed to Venezuelan crude oil for 21 or more days, and in gobies (*Zosterisessor ophiocephalus*) residing at a polluted site (elevated PCBs, PAHs, metals) in the Venice Lagoon (Payne et al. 1978; Kiceniuk and Khan 1987; Pulsford et al. 1995). Juvenile rainbow trout exposed for 24 h to a component of BKME experienced significant decreases in the SSI and hemoglobin concentration. A significant increase in the SSI and leukocrit, and significantly higher cumulative mortality after disease challenge were seen after a 25-d exposure (Johansen et al. 1994). Histological data show cellular changes occurring in the spleen with exposure to contaminants, supporting the use of the SSI as a relevant indicator of spleen dysfunction. Chronic exposure of rainbow trout to bis(tri-n-butyltin) oxide resulted in a concentration-related splenic lymphocyte depletion. Reticuloendothelial cells proliferated in the spleen, suggesting an increased need for phagocytes to remove damaged blood cells, and increased erythrophagia was noted (Schwaiger et al. 1992). Lymphoid cell depletion and cell necrosis in the spleen have been recorded in rainbow trout exposed to the fungicide triphenyltinacetate, redbreast sunfish exposed to a mixture of PCBs and metals, and rainbow trout held in Rhine River water (elevated levels of PAHs, chlorinated hydrocarbons, and metals) (Schwaiger et al. 1996; Teh et al. 1997; Poels et al. 1980). Chronic tetrachlorodibenzo-*p*-dioxin exposure caused splenic lymphoid depletion and overall splenic atrophy in rainbow trout, though recovery seemed to occur with removal to clean water (Fisk et al. 1997). Certain contaminants can affect organs such as the spleen directly (size and function) or they can suppress immune system functions (Anderson et al. 1989; Hutchinson and Manning 1996a), increasing disease prevalence and thus causing enlargement of the spleen.

Condition factor, the HSI, and the SSI appear to be useful indicators of fish health; however, these indices must be interpreted with caution. Potentially confounding factors need to be recognized when one uses such indices to compare groups of fish for the effects of contaminants. A primary restriction is that the condition factor and organo-somatic indices should only be compared within a species or between/among similar species. Also, the study protocol must be consistent and conservative in terms of sampling, particularly since the SSI and HSI can be altered within minutes by capture and holding stress. The nature of the indices may also restrict their use. For example, the HSI can only be measured on fish with discrete livers. It is simply not practical to dissect and weigh all of the liver pieces for fishes with a dispersed liver [e.g., common carp (*Cyprinius car-*

pio)] in the field. A solution to the problem of gonad size varying with season and thus affecting other indices was proposed by Grady et al. (1992), who suggested that liver size was better expressed relative to fish size exclusive of the gonads. This recommendation, which is also applicable to the condition factor and SSI, was incorporated into BEST protocols being evaluated in the Mississippi River, Columbia River, and Rio Grande basins (Schmitt et al. 1995; Bartish et al. 1997)

Value and Utility of Condition Factor and Organo-somatic Indices in the BEST Program

Although quite crude compared to many biomarkers, condition factor and organo-somatic indices are important to the BEST program. As general indicators of the overall health and well-being of the fish, alterations in these indices may indicate deleterious effects resulting from exposure to chemicals or classes of chemicals. Some of these chemicals may not even be recognized as threats because they remain undetected by current water quality monitoring protocols. The indices also integrate, at the organ system and organism level, the combined effects of multiple contaminants and the combined effects of contaminants and other stressors. A decrease in condition factor, the HSI, or both is considered a reflection of depletion in energy reserves (Goede and Barton 1990; Barton et al. 1987) because these indices are positively related to total muscle and liver energy content (Lambert and Dutil 1997). A logical link then exists between this depletion of energy reserves and potential health problems for fish. An increase in both the condition factor and HSI can, however, also signal the deleterious effect of a stressor. Although the general interpretation is that a greater weight relative to length indicates a healthier condition (for the individual organism), the presence of fewer, larger, and more robust individuals may signify an out-of-balance or abnormal condition at the population or community level (Wege and Anderson 1978; Lehtonen and Jokikokko 1995). An increase in the HSI suggests increased metabolism of xenobiotics (Fabacher and Baumann 1985) and may also be linked to pathological damage (Hinton and Laurén 1990). Depending on the relation of the condition factor and HSI to the specific energy reserves in a fish species (e.g., muscle protein, liver lipid), the condition factor and HSI may respond at different rates to environmental conditions, reflecting the different mobilization or accumulation rates of different types of stored energy. The use of condition factor and the HSI could thus provide insight into shorter- and longer-term responses of fishes to stressors (Lambert and Dutil 1997). Although differences in the HSI may indicate changes on a shorter time scale than condition factor, data from a number of studies (Grady et al. 1992; Oikari and Niittylä 1985; Holm et al. 1994) still support the use of the HSI as an indicator of chronic rather than acute or recent pollution.

Condition factor is also considered a useful indicator in monitoring because baseline data are available for comparison; length-weight equations and condition factors for many of the North American fishes likely to be sampled by the BEST program have been tabulated (Carlander 1969; Carlander 1977). Data on condition factor can also be used to compare growth of sampled fishes against established empirical standards (Goede and Barton 1990). Finally, condition factor is a recognized indicator of fish health that is being used to monitor fish populations in other national programs (Bulger et al. 1995).

The SSI is of interest due to the spleen's hematopoietic function, which also makes it an immune system organ. Alterations in relative spleen size could signal a dysfunction capable of affecting individual health. Decreased size has often been seen with acute, nonspecific stressors, but chronic exposure to a number of chemical contaminants also leads to this effect. The decrease seems to be due to necrosis and perturbations in cell processing, both of which could impact the overall condition of the individual fish. An increased SSI, on the other hand, appears to be linked to a diseased state. Disease incidence in the population may thus be estimated with this parameter. Information on alterations in all of the discussed parameters may provide an early warning of an incipient or impending problem, one of the stated objectives of the BEST program (U.S. Fish and Wildlife Service 1993). For these reasons, the National Research Council (NRC), in its review of the BEST program, strongly recommended that the program include such general indicators (NRC 1995).

Although abnormal condition factors and organo-somatic indices cannot be linked to specific causal mechanisms due to the influence of confounding factors, they do indicate perturbations in biological systems at the organismal level. Changes in the overall condition of organism(s) may corroborate findings from other biomarkers and from chemical analyses, and thereby document whole-organism and higher-level effects for those contaminants that are being measured as part of the weight-of-evidence approach inherent in the BEST program. Correlation among biomarkers at different biological and temporal scales should allow detection and quantification of

the biological impact of contaminants and may help investigators in isolating causal mechanisms (Ham et al. 1997).

THE NECROPSY-BASED FISH HEALTH ASSESSMENT

Vicki S. Blazer

Two types of assessments have been directed toward whole fish or gross (visible to the naked eye) observations: the incidence of gross external pathological disorders and a more comprehensive necropsy-based fish health assessment (internal and external). Changes at this level represent an advanced stage, i.e., when a high incidence of skin abnormalities or hepatic tumors are found there may already have been a significant impact on the population. Lesions observed at this level also suggest that adaptive mechanisms – immunological, physiological, biochemical – have been overwhelmed. However, it must be recognized that even lesions at this level can heal or be resolved and are not necessarily life-threatening.

Background

The prevalence or percentage of fish with visible pathological disorders has been used for many years as a convenient and relatively easy indicator of environmental quality by fisheries managers and field personnel. Visible lesions generally include fin erosion (Fig. 7), skin ulcers (Fig. 8), eye disorders (Fig. 9), visible tumors and skeletal deformities. The index of biotic integrity, which was designed to evaluate quality or condition of an aquatic ecosystem, includes external abnormalities in its calculation. Three categories of fish community metrics, species abundance, trophic composition, and health and abundance of fishes, are used to reflect the condition of the fish community and the environment in which it is found. Health is determined by the proportion of fish with disease or anomalies (Karr 1981).

Goede (1989) developed a systematic fish health/condition or necropsy-based system for use by fisheries personnel at the field level. It was developed to use minimal equipment to provide a rapid, relatively inexpensive method in order to detect trends in health and condition of fish populations (Goede and Barton 1990). This method is a more robust and sensitive indicator of fish health as it includes both internal and external observations and uses a computer program (AUSUM) to calculate a

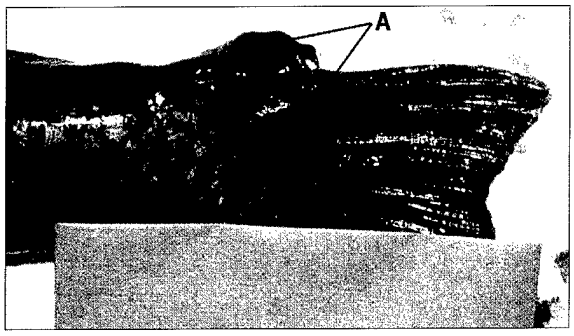


Figure 7. Erosion (A) of the caudal fin.

variety of indices to compare populations either spatially or temporally. It has been used in hatchery (Goede and Barton 1990; Novotny and Beeman 1990) and field (Goede and Barton 1990) situations. This original method did not provide a quantitative basis for comparing populations statistically. Hence, a health assessment index (HAI) and the AUSUM 430 program were developed by the Tennessee Valley Authority (TVA) primarily for use in warm water environments, and have been included in TVA's standardized aquatic biomonitoring program. This modification of the original program gives numerical ratings to the observations. It has been used in a range of reservoir types (Adams et al. 1993; Pritchard 1995) as well as river systems (Coughlan et al. 1994; Sutton et al. 2000).

Performing the Necropsy-Based Fish Health Assessment

In Goede's original method, field personnel are provided with a data sheet and accompanying description of lesions (Table 4) in order to document observations made on live fish. The information on the data sheet is entered into the AUSUM computer program and a summary of the necropsy is generated.

The quantitative health assessment index (HAI) uses basically the same variables as Goede's original method, with some additions, and substitutes a numerical value as shown in Table 5. The values are summed and an actual value is computed for each fish. These values can then be used to calculate site means and compare sites statistically. In the BEST projects, a revised data sheet was used (Schmitt et al. 1999) and substituted values as described by Adams et al. (1992b) were calculated.

Factors That Can Affect the Necropsy-Based Fish Health Assessment

Laboratory studies have demonstrated that gross

Table 4. Necropsy classification (from Goede 1989). Items in parentheses are entered onto the data sheet and into the computer program.

Length:	Total length (L) in mm
Weight:	Weight (W) in gm
CF:	$W \times 10^5/L^3$
Eyes:	Normal (N), Exopthalmia (E1, E2), Hemorrhagic (H1, H2), Blind (B1, B2), Missing (M1, M2), Other (OT)
Gills:	Normal (N), Frayed (F), Clubbed (C), Marginate (M), Pale (P), Other (OT)
Pseudobranch:	Normal (N), Swollen (S), Lithic (L), Swollen and Lithic (S&L), Inflamed (I), Other (OT)
Thymus:	No hemorrhage (0), Mild hemorrhage (1), Severe hemorrhage (2)
Fins:	No active erosion or previous erosion healed over (0), Mild active erosion with no bleeding (1), Severe active erosion with hemorrhage and/or secondary infection (2)
Opercles:	No shortening (0), Mild shortening (1), Severe shortening (2)
Mesentary Fat:	Internal fat is expressed with regard to amount present - 0 - None 1 - Little, less than 50% of each cecum covered 2 - 50% of each cecum is covered 3 - More than 50% of each cecum is covered 4 - Ceca are completely covered by large amount of fat
Spleen:	Black (B), Red (R), Granular (G), Nodular (NO), Enlarged (E), Other (OT)
Hind gut:	No inflammation (0), Mild inflammation (1), Severe inflammation (2)
Kidney:	Normal (N), Swollen (S), Mottled (M), Granular (G), Urolithic (U), Other (OT)
Liver:	Red (R), Light red (B), "Fatty" liver - "coffee with cream" color (C), Nodules in liver (D), Focal discoloration (E), General discoloration (F), Other (OT)
Bile:	0 - Yellow or straw color, bladder empty or partially full 1 - Yellow or straw color, bladder full and distended 2 - Light green to "grass" green 3 - Dark green to dark blue-green
Blood:	Hematocrit - Packed cell volume of red blood cells (erythrocytes) expressed as percent of total blood volume. Leucocrit - Volume of white blood cells (leucocytes) expressed as per cent of total blood volume ("buffy" zone of the packed cell column).

Table 5. Fish health assessment variables and substituted values (as described in Adams et al. 1992b).

Variable	Variable condition	Field Designation	Substituted Value
Thymus	No hemorrhage	0	0
	Mild hemorrhage	1	10
	Moderate hemorrhage	2	20
	Severe hemorrhage	3	30
Fins	No active erosion	0	0
	Light active erosion	1	10
	Moderate active erosion, some hemorrhage	2	20
	Severe active erosion with hemorrhage	3	30
Spleen	Normal: black, very dark red or red	B	0
	Normal: granular, rough appearance	G	0
	Nodular, containing fistulas or nodules	D	30
	Enlarged	E	30
	Other: aberration not fitting any above	OT	30
Hindgut	Normal, no inflammation or reddening	0	0
	Slight inflammation or reddening	1	10
	Moderate inflammation or reddening	2	20
	Severe inflammation or reddening	3	30
Kidney	Normal: firm, dark, flat	N	0
	Swollen: enlarged or swollen	S	30
	Mottled: gray discoloration	M	30
	Granular in appearance and texture	G	30
	Urolithiasis or nephrocalcinosis White-cream mineral deposits	U	30
	Other: aberration not fitting any above	OT	30
Skin	Normal: no aberration	0	0
	Mild skin aberrations	1	10
	Moderate skin aberrations	2	20
	Severe skin aberrations	3	30
Liver	Normal: solid red or light red color	A,B	0
	"Fatty" liver, "coffee with cream" color	C	30
	Nodules or cysts in liver	D	30
	Focal discoloration	E	30
	General discoloration	F	30
	Other: deviation not fitting any above	OT	30
Eyes	No aberration, good, clear eyes	N	0
	Opaque eye (one or both)	B	30
	Swollen, protruding (one or both)	E	30
	Hemorrhaging (one or both)	H	30
	Missing one or both eyes	M	30

Table 5 (continued).

	Other: deviation not fitting any above	OT	30
Gills	Normal: no apparent aberrations	N	0
	Frayed, ragged appearance	F	30
	Clubbed, swelling of tips	C	30
	Marginate: light discolored margin	M	30
	Pale, very light color	P	30
	Other	OT	30
Pseudobranchs	Normal, flat	N	0
	Swollen, convex in appearance	S	30
	Lithic, mineral deposits	L	30
	Swollen and lithic	S&L	30
	Inflamed, redness, hemorrhage	I	30
	Other	OT	30
Parasites	No observed parasites	0	0
	Few observed parasites	1	10
	Moderate parasite infestation	2	20
	Numerous parasites	3	30

lesions can be induced by exposure to contaminants (Couch et al. 1977; Sindermann 1979; Capuzzo et al. 1988). Field studies have also shown that fish in severely polluted areas have a higher frequency of gross lesions than in similar, less polluted habitats (Sindermann 1979; Malins et al. 1984; Malins et al. 1988; Couch 1985; O'Connor et al. 1987; Vethaak and Rheinallt 1992; Fournie et al. 1996). The vast majority of these studies have been conducted in estuarine or marine environments. The recent report by Fournie et al. (1996) provides a regional-scale perspective on the prevalence of gross abnormalities. The U.S. Environmental Protection Agency's Environmental Monitoring and Assessment Program collected fish from 120 randomly selected estuarine sites in the Virginian province (mid-Atlantic) and 220 sites in the Louisianian province (Gulf Coast) in 1991 and 1992. A total of 24,291 fish representing 143 species were examined. Skin lesions were the most prevalent gross abnormalities in both provinces, followed by ocular abnormalities in the Virginian province and branchial chamber abnormalities (gill parasites, gill arch deformities; Fig. 10) in the Louisianian province. Prevalence, in general, was three-fold higher for demersal fishes than pelagic and eight-fold higher at sites with high sediment contaminant concentrations.

Although contaminants alone can induce grossly visible lesions in the laboratory, it must be recognized that more often development of gross

Figure 8. Ulceration of the skin (A).

Figure 9. A missing or scarred-over eye (A).

lesions requires a set of interacting conditions. These include susceptible (or sensitive) hosts, stressors, and infectious disease organisms. Hence, genetics, species, food habits, contaminants, other water quality parameters, weather, handling or capture methods, trauma of other types, and the presence and number of disease organisms play a role in the development of gross lesions.

Value and Utility of the Necropsy-Based Fish Health Assessment in the BEST Program

Necropsy-based assessments were developed to be performed by field personnel with minimal training, equipment, and expenses and to provide a rapid indication of the health of the population being studied. They are not intended to be diagnostic but rather to alert investigators to possible problems that should then be investigated with more diagnostic (specific) tests. It must be recognized that these methods are not biomarkers but rather methods for documenting lesions or changes that have advanced to the point of being grossly visible. The value of the necropsy-based HAI is: 1) it provides a systematic method for field personnel to observe and document gross abnormalities; 2) it allows investigators to compare incidences of grossly observable lesions (those the public is most concerned about) among sites; and, 3) it allows investigators to document both spatial and temporal trends. The drawback of this method, if used alone, is it is not necessarily an early warning system and does not generally provide an indication of causes. Nevertheless, it does estimate cumulative stress and, within the BEST program, it is used in concert with other biomarkers to assess environmental conditions.

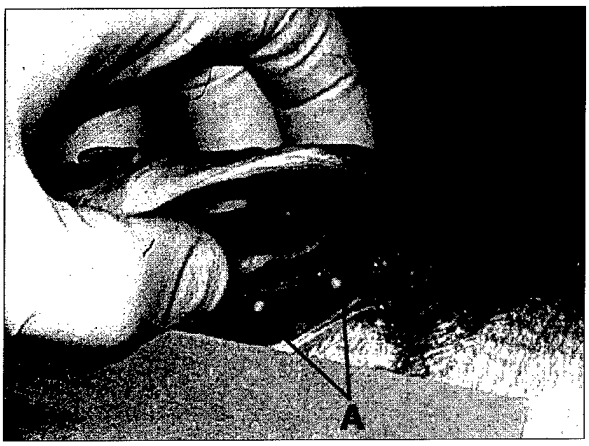


Figure 10. Parasites (A) on the gill.

HISTOPATHOLOGIC ASSESSMENT

Vicki S. Blazer

Histopathology is the study of lesions or abnormalities on a cellular level. Organs and tissues from fishes of any size, age or type can be examined. When organs or tissues are properly fixed after use in the necropsy-based health assessment, they can be stored until later processing. Sectioning of these fixed tissues allows retention of *in vivo* relationships. More than one tissue may be studied simultaneously to determine biological effects of toxicants not only in localized portions of certain organs but subsequent derangements in tissues or cells at other locations. This often allows for diagnoses of changes observed grossly as well as indications of mechanisms of toxicity. In addition, cellular changes that occur prior to development of grossly visible lesions can often be detected. Macroscopic signs of toxicity are almost always preceded by changes at the tissue, cellular or molecular levels (Segner and Braunbeck 1990). When the concentration of a toxicant(s) is not sufficient to cause acute death there may be sublethal or adaptive changes which occur. When cell injury or death of cells without the death of the organism occurs, this is followed by cellular reactions and/or host responses that can be described and sometimes be diagnostic of cause(s).

Background

Histopathology has been used for many years to study the cellular basis of infectious and noninfectious diseases. Fish respond to various insults in ways very similar to mammals. Therefore, fish histopathology utilizes knowledge gained over many years in human and veterinary pathology. To perform histopathologic

analyses, the pathologist must know the normal structure of fish tissues – their unique tissue types and responses. Fortunately, there are a number of excellent descriptions available for selected species (Ashley 1975; Grizzle and Rogers 1976; Groman 1982; Kubota et al. 1982; Yasutake and Wales 1983; Ferguson 1989). Histopathologic changes observed in infectious and noninfectious fish diseases have also been documented in a number of sources (Ribeln and Migaki 1975; Kubota et al. 1982; Ellis 1985; Ferguson 1989; Roberts 1989; Sindermann 1990).

It has been recognized for many years that fish respond to toxicant exposure, sometimes in fairly specific ways (Braunbeck 1994), but often the same response is elicited by a variety of chemicals or chemical mixtures. There have been a number of excellent reviews directed toward evaluation of histopathologic changes as biomarkers of contaminant exposure (Hendricks et al. 1985; Hinton et al. 1992; Hinton 1993). Hinton et al. (1992) divided histologic biomarkers into present and future. Present biomarkers are reliable, well-documented lesions evaluated both in laboratory and field situations. Future or potential biomarkers are those which may have strong field relevance but have not been laboratory-validated or which have been observed in laboratory exposures but have not been evaluated in the field. It should be recognized that as information is collected from both field and laboratory studies future biomarkers may become present biomarkers. For the organs routinely examined in the BEST program only the liver and reproductive tract have what are considered present biomarkers by Hinton et al. (1992).

Performing Histopathological Analyses

Pieces of any organ (or whole small fish) are collected in the field and placed in fixative (Fig. 11). It is very important to have sufficient fixative for the volume of tissue. If whole fish are fixed, it is very important to slit open the abdominal cavity so fixative can penetrate all organs. A variety of fixatives, including neutral buffered formalin, NoTox®, Davidson's (Dietrich's) and Bouin's (Luna 1992) have been used. Each fixative type has advantages and disadvantages. Once properly fixed, tissues can be stored for long periods of time at room temperature. Routine processing of tissue involves trimming into small pieces, dehydration through a series of alcohols followed by an organic solvent, and infiltrating with paraffin. Blocks of paraffin containing the tissues are allowed to harden and then cut into 3 – 6 µm slices. These sections are placed on glass slides, allowed to dry, deparaffinized, and stained. The most commonly used stain is hematoxylin and eosin (H&E). A variety of other staining techniques are used to demonstrate infectious agents, cellular components or specific pathological responses (Luna 1992).

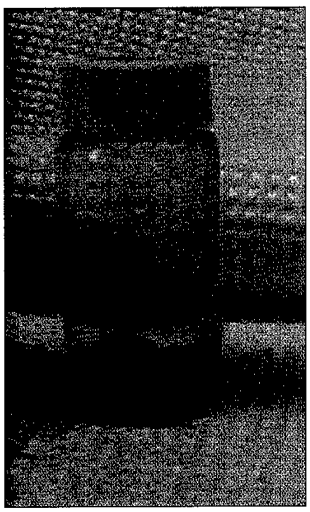
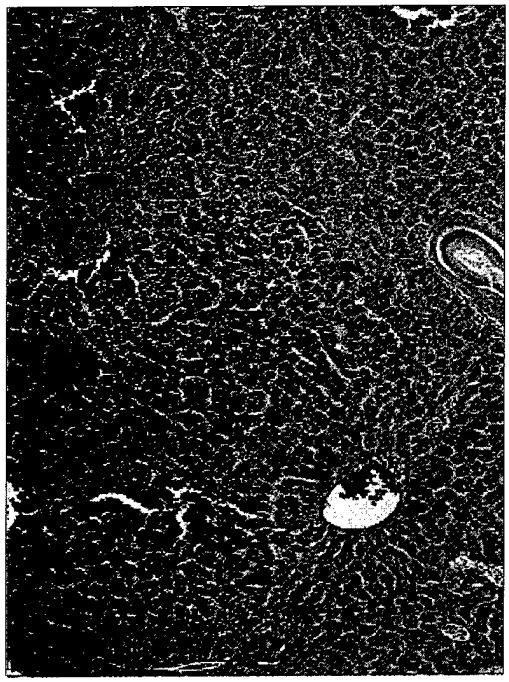


Figure 11. Tissue in fixative.

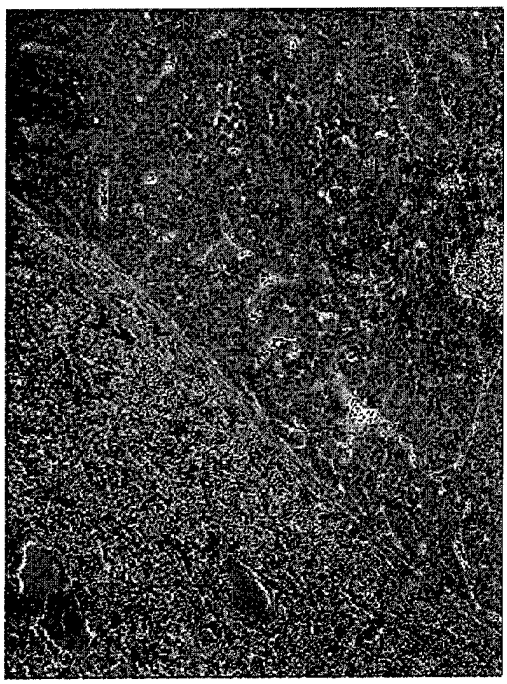
Examination of tissue sections should be done by an experienced histopathologist with knowledge of normal fish histology as well as an understanding of pathology. Many histopathological studies rely on descriptive comparisons. Few, to date, have used quantitative methods that can then be compared statistically. Reimschuessel et al. (1992) described a classification system that allows for a statistical comparison among groups. Lesions or observations are classified according to location, type of change (inflammatory, necrotic, growth), extent (focal, multifocal, diffuse) and severity (rated on scale of 1-4 or 5). Hence, a hepatocarcinoma would be classified as DI.LI GC.NE.MA M 3 (Digestive System.Liver Growth Change.Neoplasia.Malignant Multifocal 3=moderate). If only one small focus of neoplastic tissue was observed it may receive a 1 or 2 severity rating. Use of this system allows comparisons of prevalence as well as severity among sites.

Factors That Can Affect Histopathological Biomarkers

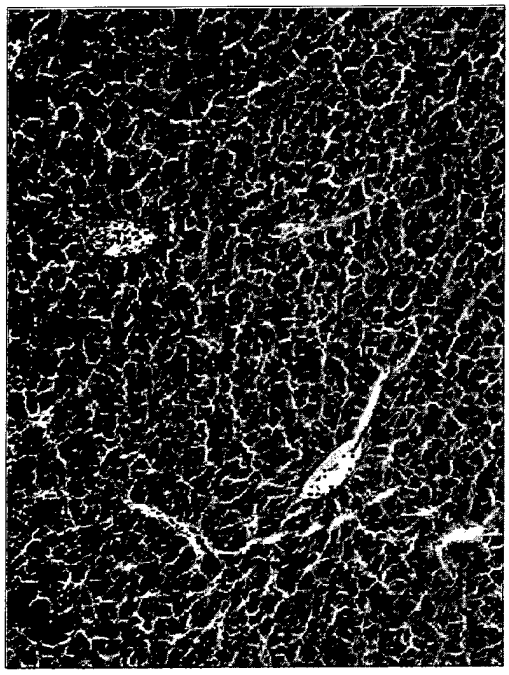
The liver is, by far, the most studied organ in terms of toxic effects. The liver is the site of detoxification mechanisms such as the mixed function oxidase system and metallothioein; it produces many regulatory proteins; it is the site of energy storage – both lipid and glycogen; and it synthesizes bile. Present hepatic biomarkers include: hepatocellular coagulative necrosis, hyperplasia of regeneration, bile ductular/ductal hyperplasia, hepatocytomegaly (hepatocellular hypertrophy), foci of cellular alteration, and hepatic and bile duct neoplasms (Fig. 12). These have all been well described by Hinton and Laurén (1990), Hinton et al. (1992), and Hinton (1993), and contaminants that cause these changes have been described by the same authors. In laboratory exposures, species (Braunbeck et al. 1990), sex (Braunbeck et al. 1989),



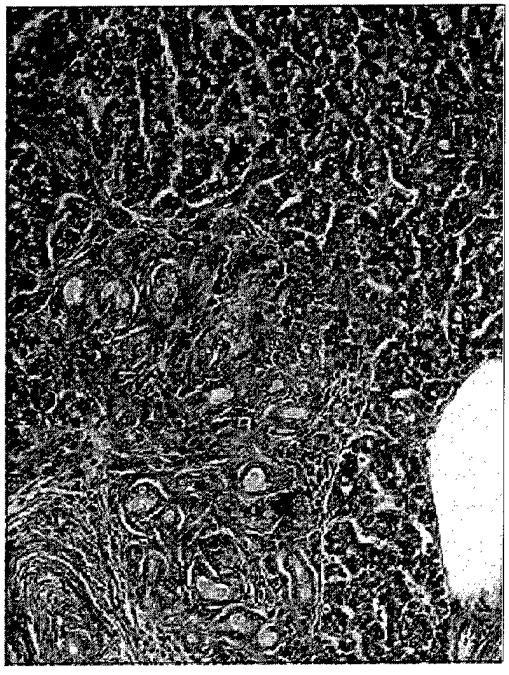
Basophilic foci (A); rainbow trout



Large tumor (A) compressing normal tissue; rainbow trout



Normal liver, rainbow trout



Abnormal bile duct proliferation (A); rainbow trout

Figure 12. Examples of histologic biomarkers in the liver (165x).

and age (Braunbeck et al. 1992) have all been shown to affect hepatocellular reactions to chemicals. Because the liver is also the site of energy storage — i.e., glycogen and/or lipids — many other factors such as nutritional status, season, and food habits will affect the gross and histologic appearance of the liver.

The other present biomarker used in the BEST program is oocyte atresia. Oocyte atresia is degeneration and necrosis of developing ova. It is normal for ovaries of fish to resorb ova that are not released. However, it can become pathologic following exposure to certain contaminants. Oocyte atresia is discussed in 'Reproductive Indicators'.

The kidney has varied functions among fish species. However, the kidney does filter large quantities of blood and produce urine, which is a major route of excretion for some xenobiotics. In at least some species of fish, as in mammals, the kidney can regenerate after sublethal toxic damage (Reimschuessel et al. 1990). Hence, development of new nephrons has recently been suggested as a possible biomarker of exposure. Histologically these new nephrons first appear as basophilic clusters. The developing nephrons also stain more basophilic and the number of these structures can be quantified per square millimeter of tissue. These have been shown to increase following laboratory exposures of goldfish (*Carassius auratus*) to hexachlorobutadiene (Reimschuessel et al. 1990), mercury (Reimschuessel and Gonzalez 1992), and gentamicin (Reimschuessel et al. 1991) and of rainbow trout (*Oncorhynchus mykiss*) to tetrachloroethylene (Reimschuessel et al. 1994). Cormier et al. (1995) evaluated this biomarker in field situations. They found significantly increased numbers in Atlantic tomcod (*Microgadus tomcod*) from a contaminated site (high levels of polychlorinated biphenyls and pesticides) when compared to one reference site, but not a second reference site. In brown bullheads (*Ameriurus nebulosus*) collected from the Cuyahoga River (Ohio) with high levels of polycyclic aromatic hydrocarbons, developing nephrons were significantly higher than the reference sites. These authors suggested that developing nephrons may have potential as general indicators of environmental condition but may have more utility for detection of specific nephrotoxins. A significant increase in developing nephrons was observed in white suckers (*Catostomus commersoni*) collected in a contaminated reach of the Sheboygan River (Wisconsin) when compared to a reference area (Schrank et al. 1997). Contaminants known to be problems in this area include polychlorinated biphenyls, polycyclic aromatic hydrocarbons and heavy metals.

In the spleen, macrophage aggregates are a histologic feature, and alterations in their number, size, or both have been suggested as potential biomarkers. These structures are described in "Immune System Indicators."

Value and Utility of Histopathological Biomarkers in the BEST Program

The use of histopathology of selected organs together with the other fish health assessment methods allows for a more comprehensive evaluation of abnormalities than grossly visible alone. In many situations the cause(s) of lesions observed grossly can be determined, and a judgment as to whether these are contaminant- or disease-related can be made. In addition, changes not yet visible to the naked eye can be documented. Hence, histology offers a "very early warning" system for potential contaminant effects. In addition, there have been few large-scale, regional or river system type evaluations of histologic lesions. Those that have been conducted have primarily focused on estuarine or marine fishes (Sindermann 1990; May et al. 1987; Myers et al. 1994). Thus, development of this type of database will provide baseline comparisons for other studies, provide needed field information for validation of selected biomarkers, and provide additional information on species, sex, and age as confounding factors.

IMMUNE SYSTEM INDICATORS

Vicki S. Blazer and Gail M. Dethloff

Immunotoxicology is a relatively new and emerging branch of environmental toxicology. In the early 1970s it became apparent that chemicals known to be present in the environment could compromise immunity in animals. In the 1980s it was confirmed that a variety of environmental contaminants such as toxaphene (Allen et al. 1983), lead, polychlorinated biphenyls (PCBs) (Koller et al. 1983), and pentachlorophenol (Kerkvliet et al. 1982) produced immunosuppression at dosages lower than those that altered other commonly used toxicological indices (Koller 1996).

The immune system of fishes has been shown to be as sensitive to a variety of environmental contaminants (Weeks et al. 1992; Wester et al. 1994;

Zelikoff 1994) as that of homeotherms. For this reason there is strong interest in incorporating measures of immunity and disease resistance for fish into the overall organism health assessments conducted as part of the BEST program. It is important to note, however, that this represents an emerging area of aquatic toxicology, and both the methods and the significance that can be attached to their results are developing rapidly. Generally, assessment of immune system function requires harvesting live cells (macrophages or lymphocytes), maintaining them at optimal conditions in sterile liquid media, and performing functional assays within a short period of time. Most of the functional assays require sterile techniques, sophisticated equipment, and specially trained personnel, and often utilize radio-labeled substrates. Although these types of assays have been used in field studies of limited scope, they are simply not feasible in large-scale programs such as the BEST projects. Consequently, Weeks et al. (1992) suggested a tiered approach for use in screening or comprehensive analysis of immunomodulatory effects of chemicals on aquatic organisms. Organosomatic indices, histology of spleen and lymphoid tissue, lysozyme activity, and macrophage aggregate analyses were all included in tier 1. These are methods that can be performed on cryogenically frozen plasma or serum (lysozyme) or with preserved tissue (histology of lymphoid tissue; macrophage aggregate parameters) and were chosen as biomarkers for the BEST program.

Serum/Plasma Lysozyme Activity

Lysozyme is an enzyme with antibacterial and antiviral activity (Jolles and Jolles 1984) that acts on peptidoglycan (a protein in bacterial cell walls), causing lysis of the bacteria (Chipman and Sharon 1969). Gram-positive bacteria are most susceptible to its action; however, rainbow trout (*Oncorhynchus mykiss*) lysozyme has been shown to lyse a number of gram-negative bacteria (Grinde 1989). It is believed that the lysozymes of both fishes and mammals work in conjunction with other proteolytic enzymes to lyse gram-negative organisms (Neeman et al. 1974; Hjelmeland et al. 1983). Lysozyme is an enzyme of leucocytic origin considered to be an important component of the nonspecific humoral disease resistance mechanisms of fishes (Yano 1996). It has also been found to increase with enhanced phagocytic function of macrophages in rats and therefore has been suggested as a marker for macrophage function (Kokoshis and Di Luzio 1979). However, this has not been validated with fishes.

Background

Lysozyme is a disease resistance factor that has been studied for many years, particularly in relation to infectious diseases and vaccination of cultured fishes. Serum/plasma lysozyme levels have been shown to rise in the early stages of coccidial infections of common carp (*Cyprinus carpio*), peaking at the point clinical lesions are evident and then declining slightly as the infection progresses (Studnicka et al. 1986). Common carp immunized with the bacteria *Aeromonas punctata* (Vladimirov 1972) or experimentally infected with a mixture of *A. punctata* and *Pseudomonas alcaligenes* (Siwicki and Studnicka 1987) exhibited a rise in circulating lysozyme. This has also been shown for Atlantic salmon (*Salmo salar*) inoculated with the bacterium *Vibrio anguillarum* (Muona and Virtanen 1993) or infected with *Aeromonas salmonicida* (Møyner et al. 1993). Recently, there has been some interest in using lysozyme activity as an indicator of environmental stress with the potential of linking alterations in lysozyme to disease prevalence. However, the use of lysozyme in either field studies or contaminant-related laboratory studies has been limited.

Performing the Lysozyme Assay

Blood is collected from living fish and chilled, then allowed to clot or centrifuged to obtain either serum or plasma respectively, depending on study objectives and protocol. Plasma or serum is quick-frozen in liquid nitrogen or dry ice and stored cryogenically (-80°C) until needed for analysis. Measurement of lysozyme activity is based on the lysis of suspensions of the bacteria *Micrococcus lysodeikticus* by serum/plasma. Variations of a photometric method (Litwack 1955) and of a lysoplate assay described by Osserman and Lawlor (1966) have both been used with fish serum. The pH optima for the conduct of the assay vary with taxon and should be determined in advance for the species under consideration (Möck and Peters 1990; Blazer et al. 1996). Lysozyme from the whites of chicken (*Gallus gallus*) eggs is generally used to produce a standard curve. However, the optimum of hen egg white lysozyme is pH 7.5 (Möck and Peters 1990) while fish serum optima generally range from 5.5 (Möck and Peters 1990; Blazer et al. 1996) to 8.0 (Kusuda et al. 1987). Investigators have recognized this difference in pH optima among fishes; however, there is no consistency in approaches to deal

with the difference. Many investigators use the same buffer system for both standard and unknowns (Ellis 1990; Tahir et al. 1993), while others have suggested the use of an internal standard (Möck and Peters 1990; Røed et al. 1993). We chose to use a kinetic method, in which groups of fish are compared using mOD/min or the change in absorbance over a specified time.

The microplate method developed by Tahir et al. (1993) and modified by Blazer et al. (1996) was used to analyze the plasma of fish from the BEST projects (Schmitt et al. 1995; Bartish et al. 1997). A 0.075% suspension of dried *M. lysodeikticus* is prepared in the appropriate buffer. Serum or plasma (25 µl) is added (triplicate determinations for each fish) to wells of a 96-well, flat-bottomed micro-titer plate. A 175-µl aliquot of the above suspension is then added, and the plate is immediately shaken and read on a kinetic microplate reader at 450 nm every 15 seconds over a 5 minute period.

Factors That Can Affect Serum/Plasma Lysozyme Activity

As noted above, disease can alter lysozyme levels. Water temperature appears to have a positive correlation with lysozyme (Balfry et al. 1997; Aranishi et al. 1998). Lysozyme activity has also been found to be modulated by certain dietary factors (Kiron et al. 1995; Roberts et al. 1995). In addition, seasonal, sexual, species, strain and age-dependent variations in plasma lysozyme activity have been reported (Balfry et al. 1997; Holloway et al. 1993; Fletcher and White 1976; Fletcher et al. 1977; Studnicka et al. 1986; Lie et al. 1989; Røed et al. 1993). Fish species that undergo smoltification experience altered lysozyme activity during this developmental stage (Muona and Soivio 1992).

General stressors such as handling and transport can affect lysozyme activity (Möck and Peters 1990; Fevolden et al. 1994). However, the effects appear to depend on the type or duration of the stress. Möck and Peters (1990) reported that 30 min of handling caused either a decrease or increase in rainbow trout lysozyme activity. Demers and Bayne (1997) reported an increase in the lysozyme levels of rainbow trout with short-term air exposure. Transport stress lasting 2 hours or acute exposure to un-ionized ammonia levels of 0.450mg/l, however, caused a significant decrease in lysozyme activity in rainbow trout (Möck and Peters 1990). Differing responses to short- and long-term stressors were also seen in common carp (Hajji et al. 1990) and Atlantic salmon (Røed et al. 1993). In dab (*Limanda limanda*), transport stress (approximately 60 minutes) resulted in significantly decreased serum lysozyme activity (Hutchinson and Manning 1996b).

Investigations have recently begun into the effects of contaminants on lysozyme activity. In laboratory studies, lysozyme activity of dab was reported to decrease after exposure to oil-contaminated sediments from drilling sites (Tahir et al. 1993). Also, common carp exposed to the organophosphate insecticide trichlorphon (Siwicki et al. 1990) had decreased lysozyme activity. Field studies have found reduced lysozyme activity in common carp exposed to sewage effluent (Price et al. 1997) and in European plaice (*Pleuronectes platessa*) collected along a sewage gradient (Secombes et al. 1995). However, dab exposed to sewage sludge in the laboratory for 12 weeks did not display significantly altered lysozyme activity (Secombes et al. 1991).

Value and Utility of Serum/Plasma Lysozyme Activity in the BEST Program

Despite its limited use in environmental toxicology, serum/plasma lysozyme activity remains attractive for use in the BEST program because it is among the few logistically reasonable markers of immune system function. For this reason, lysozyme activity has been included among the indicators of fish health as a general indicator of exposure to a wide variety of factors (including contaminants) not otherwise accounted for by more specific measures. However, it must be recognized that a great deal more field and laboratory data need to be generated to validate this biomarker.

Macrophage Aggregates

Pigment-bearing macrophages are a prominent feature in fish spleen, kidney and sometimes liver (Agius 1980). In advanced teleosts they form discrete aggregations called macrophage aggregates (MA) or melanomacrophage centers (Fig. 13). Macrophage aggregates are believed to be functional equivalents of the germinal centers, active in the centralization of foreign material and cellular debris for destruction, detoxification or reuse, the storage of exogenous and endogenous waste products, the immune response, and iron storage and recycling (Ferguson 1976; Ellis et al. 1976). The subject of MAs has recently been thoroughly reviewed by Wolke (1992) and by Blazer et al. (1997).

Background

Macrophage aggregates have been recognized as normal structures of fish spleen, kidney and liver since the 1920s (Jolly 1923; Yoffey 1929). Roberts (1975) coined the term melanin-macrophage or melano-macrophage centers and suggested these structures should be considered part of the reticulo-endothelial system and hence part of the defensive system against infectious disease. It has been shown that particulate matter (such as carbon particles or bacteria) injected intraperitoneally or intravascularly eventually accumulates within these aggregates or centers (Mackmull and Michels 1932; Ellis et al. 1976). It is also known that bacteria and parasites may be phagocytized and taken to these centers (Roberts 1975; Ferguson 1976; Herraez and Zapata 1987). Agius was the first to study these structures in depth (reviewed in Agius 1985). He examined their phylogenetic development (Agius 1980), their ontogeny and age-related changes (Agius 1981), the effects of starvation on these structures (Agius and Roberts 1981), their role in iron storage (Agius 1979), and the development of pigments within these centers (Agius and Agbede 1984).

Wolke et al. (1981, 1985a) first suggested pigmented macrophage accumulations as potential monitors of fish health. Numerous studies had documented an increase in their number, size or hemosiderin content in fish collected at contaminated sites when compared to those collected at reference sites. For this reason, MAs have been suggested as potentially sensitive biomarkers of contaminant exposure. Although they are structures observed histologically, it has been suggested that MAs may be immunotoxicologic biomarkers (Weeks et al. 1992; Blazer et al. 1997).

Measuring Macrophage Aggregate Parameters

Macrophage aggregates are measured in preserved tissues. They can be found in spleen, kidney and liver depending on the fish species. Pieces of spleen, liver or kidney are routinely processed for histology, embedded in paraffin, cut into 5-µm sections with a microtome, mounted on glass slides, and stained with hematoxylin and eosin (H & E). A special staining procedure called the Perl's method (Luna 1992) is used to increase the ease in visualizing these structures and also allows observation of all the pigments within the MA. With this stain, melanin, the melanosome pigment derived from tyrosine metabolism, is black; hemosiderin, a protein-bound iron pigment, is blue; and ceroid/lipofuscin, lipogenic pigments arising from the oxidation of unsaturated lipids, are yellow-tan (Fig. 14). Macrophage aggregate parameters are measured using a computer-based image analysis system. The number of aggregates in 2 sq. mm of tissue and their mean size are measured. From this information the % of tissue occupied by MAs can be calculated. Currently, qualitative observations are made on pigment content. However, the relative measurement of pigments is possible with true color image analysis. A number of studies have compared the utility of splenic and hepatic MAs as indicators of contaminant exposure, and splenic MAs

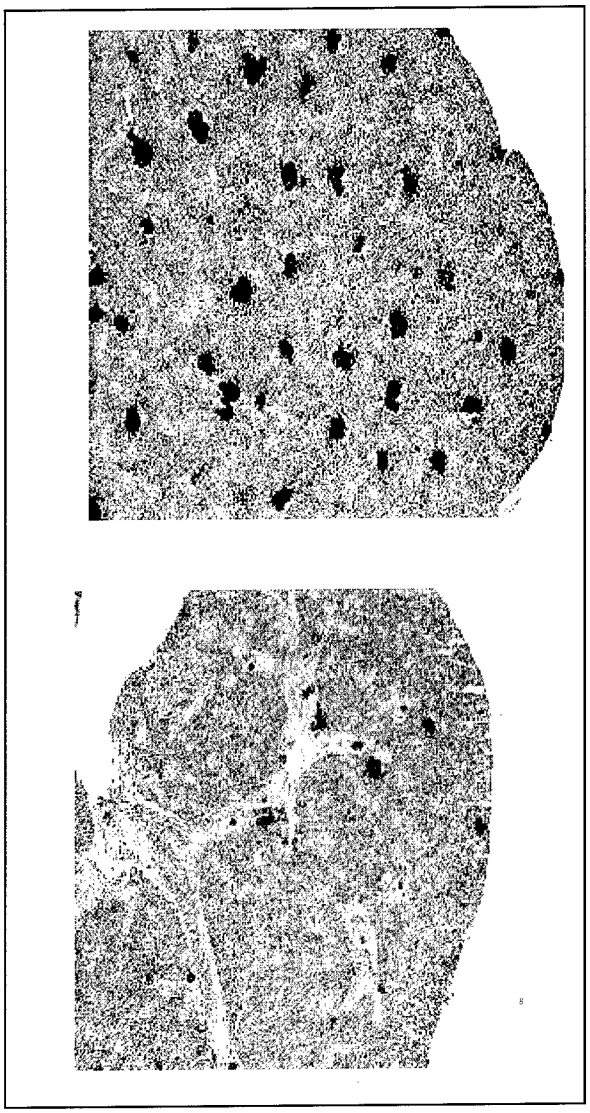


Figure 13. Splenic tissue (165x). Top panel: High density of macrophage aggregates. Bottom panel: Low density of macrophage aggregates.

have been found to be the most responsive (Blazer et al. 1994b). Therefore, in the BEST projects splenic MA parameters were measured.

Factors That Can Affect Macrophage Aggregates

Occurrence of MAs may vary depending on the size, nutritional status, or health of a particular fish species (Agius 1979; Agius 1980; Agius and Roberts 1981; Wolke et al. 1985b). Larger fish, fish with nutritional deficiencies, or fish in poor health tend to have more or larger MAs. In addition, the number and/or size of MAs increase with age (Brown and George 1985; Blazer et al. 1987).

Relatively few studies on the response of fish MAs to contaminants under controlled laboratory conditions have been reported. The majority have found that MAs increase with exposure to contaminants. Exposure of goldfish (*Carassius auratus*) to phenylhydrazine caused extensive hemolysis and resulted in significant increases in MA number and size (Herraez and Zapata 1986). Laboratory exposure of Atlantic cod (*Gadus morhua*) to the water soluble fractions of two types of crude oil caused an increase in MAs (Khan and Kiceniuk 1984). An increased number of MAs was also seen in dab exposed to sewage sludge (Secombes et al. 1991) and common carp exposed to sediments contaminated with polychlorinated dibenzodioxins/dibenzofurans (PCDD/Fs) and PCBs (van der Weiden et al. 1993). European plaice exposed to water-borne potassium dichromate had an increased MA number but a decrease in the mean size of the aggregates. In addition, there was an increase in the density of the melanin pigment (Kranz and Gerken 1987). In striped bass (*Morone saxatilis*) exposed to dietary arsenic, splenic MA number increased with increasing concentrations of arsenic. In addition, there was a striking increase in hemosiderin in fish fed higher levels (200 ppm) of arsenic (Blazer et al. 1997). In contrast, juvenile yellowfin sole (*Pleuronectes asper*), rock sole (*Pleuronectes bilineatus*) and Pacific halibut (*Hippoglossus stenolepis*) experienced decreased hepatic MA numbers with exposure to hydrocarbon-contaminated sediments; the decrease in MA numbers may, however, have been linked to significantly decreased growth in the exposed fishes (i.e. metabolism) (Moles and Norcross 1998).

A larger number of studies have noted changes in MA number in liver, spleen or kidney of fish from polluted waters (Poels et al. 1980; Bucke et al. 1984; Khan and Kiceniuk 1984; Kranz and Peters 1984; Wolke et al. 1985b; Spazier et al. 1992). Very consistently, MA numbers have been reported to increase at contaminated sites versus reference sites. There are a few studies that have suggested a decrease or no significant effect. Payne and Fancey (1989) reported a decrease in hepatic MAs of winter flounder (*Pleuronectes americanus*) after 4 months of laboratory exposure to hydrocarbon-contaminated sediments, when compared to nonexposed. However, there was no information on fish size/age, condition prior to exposure, growth or weight loss during the experiment. Haaparanta et al. (1996) compared perch (*Perca fluviatilis*) and roach (*Rutilus rutilus*) from four lakes with a gradation of water quality. Three of the lakes were polluted by effluent from a paper and pulp mill. These authors reported no observed patterns of splenic, hepatic or kidney MAs in terms of rankings of water quality. However, again methodology may play a role, as age of fish was not considered and size distribution of captured fish was different among the lakes. Conversely, Couillard and Hodson (1996) found increased density of MAs in white suckers (*Catostomus commersoni*) to be a useful marker of bleached-kraft mill effluent. In winter flounder from eight coastal embayments, the splenic area occupied by MAs was correlated with chemical contamination of sediments: PCBs, polycyclic aromatic hydrocarbons, and trace metals measured at these sites all correlated with MA parameters and the number of MAs and their size increased with increasing levels of benzo(*a*)pyrene (Benyi et al. 1989; Gardner et al. 1989). A study comparing a number of biochemical and physiological indicators of contaminant stress to population level responses of redbreast sunfish

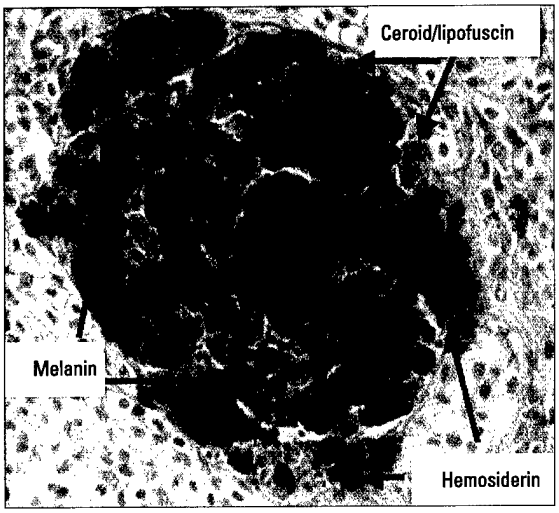


Figure 14. A macrophage aggregate in a tissue section stained by the Perl's method demonstrating all three pigments that may be present (1680x).

(*Lepomis auritus*) sampled four sites along a PCB and mercury gradient in a stream receiving point-source discharges. At the sites sampled along this gradient, MAs were more sensitive at indicating contaminant effects than cytochrome P450 and NADPH-cytochrome c reductase levels, liver-somatic index, fecundity, and population abundance and were as sensitive as serum triglyceride levels and the liver RNA:DNA ratio (Adams et al. 1992b). It is interesting to note that in a subsequent study at these same sites, macrophage function was also significantly depressed at the first three sites (Rice et al. 1996). Blazer et al. (1994a) also found an increase in MA number and percent of tissue occupied by MAs in species from contaminated sites when compared to less contaminated sites. MAs of gafftopsail catfish (*Bagre marinus*) were the best indicators of elevated tissue contaminants whereas MAs of both gafftopsail catfish and spot (*Leiostomus xanthurus*) were good indicators of elevated sediment contaminants.

Value and Utility of Macrophage Aggregates in the BEST Program

Macrophage aggregates have long been recognized as potentially useful biomarkers (Wolke et al. 1985a) but intrinsic and extrinsic factors may confound investigations into the role of contaminants. Hence, it has been recognized that continuing field and laboratory studies are needed to fully understand the relationship of MA number, size, and pigment content to various environmental contaminants (Hinton et al. 1992; Wolke 1992; Blazer et al. 1997). Nevertheless, this histologic as well as potential immune system biomarker, although quite general, has been shown through both field and laboratory studies to respond to exposure of fish to a variety of contaminants of concern. In addition, the assay is logistically reasonable as part of the suite of health assessment indicators because it is based on preserved tissues collected as part of an overall procedure.

REPRODUCTIVE INDICATORS

Kelly K. McDonald, Timothy S. Gross, Nancy D. Denslow, Vicki S. Blazer

To predict the potential of a fish or wildlife population to persist within a given ecosystem, it is necessary to evaluate the reproductive health and capacity of the individuals within that population. Multiple definitions are available for "reproductive success," including the number of eggs in a clutch, number of viable offspring, or number of viable offspring capable of reproducing. Whereas reproductive success can be a reliable indicator of a population's reproductive health and potential, it is often difficult to evaluate, especially in aquatic environments where fish and other wildlife are not easily contained for convenient monitoring. Consequently, the development of techniques for measuring other reproductive indicators (e.g., sex steroid hormones, vitellogenin, gonadosomatic indices, and gonadal histopathology) has aided researchers in assessing the reproductive health of many fish species. These reproductive indicators, or biomarkers, provide quantifiable measures of biochemical, physiological, or histological changes that occur naturally throughout the reproductive cycle. These indicators may also be altered by exogenous factors and may reflect reproductive impairment. Reproductive biomarkers are useful for (1) examining the effects of environmental stressors, such as chemical contaminants, eutrophication, and temperature fluctuations, on an individual or population, (2) predicting future reproductive trends and population abundance by serving as early indicators of sublethal effects of environmental stressors, and (3) providing insight into the causal relationships between reproductive failure and environmental stressors. For an accurate understanding of the reproductive health of an individual or population, it is necessary to measure a variety of responses using multiple methods.

The complex process of vertebrate reproduction includes sexual differentiation, embryonic development and birth or hatching, sexual maturation, gametogenesis, mating, and fertilization. Fish display a wide repertoire of reproductive strategies; however, strict hormonal and environmental regulation is required for the reproductive processes of all species. Hormonal regulation is the function of the endocrine system, a collection of cells, tissues, and organs that produce and secrete hormones that influence virtually every stage of the lifecycle (reviewed by Norris 1997; van der Kraak et al. 1998). The coordinated reproductive efforts of the hypothalamus, pituitary, and gonads have led to the designation of the hypothalamo-hypophysial (pituitary)-gonadal axis. The hypothalamus exerts ultimate control over the reproductive process by synthesizing and releasing gonadotropin-releasing hormones (GnRHs) that stimulate the release of several gonadotropin hormones from the pituitary (Fig. 15). In nonmammalian vertebrates, including several orders of teleosts, two gonadotropin hormones, GTH-I and GTH-II, have been characterized (Kawauchi et al. 1989; Swanson et al. 1991) and

their functions compared to the mammalian follicle-stimulating hormone and leutinizing hormone, respectively (Redding and Patino 1993). In fish, GTH-I is important for vitellogenesis, the process of yolk protein synthesis, and early gonadal development. GTH-II, on the other hand, is secreted late in gonadal development and plays a role in the final maturation and release of mature gametes (ovulation in females, spermiation in males). As mentioned earlier, a variety of environmental factors, including photoperiod, temperature, salinity, dissolved oxygen concentration, water flow, and turbidity, influence each stage of reproduction (reviewed by Donaldson 1990; Munro et al. 1990). Some of these environmental factors will be addressed for each parameter.

Sex Steroids

The sex steroids, a class of hormones derived from cholesterol and synthesized by the gonads in response to circulating levels of GTH-I and GTH-II (Fig. 15), collectively control the development of the gonads and gametes, secondary sexual characteristics, and reproductive behavior such as pheromonal attraction, spawning, and parenting (Liley and Stacey 1983; Fostier et al. 1983). The reproductive hormones can be divided into two major categories: (1) the androgens, produced by the testes and ovaries; and (2) estrogens and progesterone, which are primarily produced by the ovaries but are often synthesized in smaller amounts by the testes. Although species and maturational stage largely influence the type of reproductive hormones synthesized by the gonads, the major androgens in fish include testosterone, 11-ketotestosterone, and androstenedione. The predominant estrogens are 17β-estradiol and estrone. Sex steroids in immature fish probably influence gonadal differentiation, whereas these same hormones play an important role in gametogenesis, ovulation, and spermiation in mature fish (Redding and Patino 1993; Barry et al. 1990; Patino and Thomas 1990a; Patino and Thomas 1990b).

Background

Measuring reproductive hormones in plasma may yield biochemical information concerning the reproductive status of an individual, as well as provide a method for detecting potential reproductive injury. Although this technique has been used to gain information regarding early development and the reproductive cycles of healthy individuals (Johnson and Casillas 1991; Freund et al. 1995), monitoring sex steroid concentrations has primarily been utilized to study the endocrine-disrupting effects of various environmental chemicals. Early evidence from studies of fish-eating birds suggested that some chlorinated hydrocarbons impaired normal cyclical changes in plasma concentrations of estrogen, progesterone, and testosterone (Peakall et al. 1981). Over the last few decades, sex steroid hormones have evolved as convenient biomarkers for detecting contaminant-induced biochemical alterations, and fish have emerged as favorable models for examining the effects of environmental pollutants in aquatic ecosystems.

Performing Sex Steroid Hormone Assays

Due to its facility and inexpensiveness, the method utilized by the BEST program involves the analysis of sex steroid hormones (e.g., 17β-estradiol, testosterone, 11-ketotestosterone) in serum or plasma samples by radioimmunoassay (Goodbred et al. 1997). Using this technique, a given hormone concentration in a serum or plasma sample can be determined. The samples are extracted twice with an organic solvent (e.g., ethyl ether) to isolate lipophilic compounds such as sex steroids. The reaction solution, comprised of the serum or plasma extract, a radiolabeled hormone (e.g. ^{3}H-estradiol), and a corresponding hormone-specific antibody, is allowed to equilibrate overnight. During this time the unlabeled hormone from the extract and a constant concentration of the corresponding radiolabeled hormone compete for the same antibody binding sites. The non-antibody bound, radiolabeled hormone is then removed from solution by addition of charcoal dextran followed by centrifugation. The resulting solution, containing bound radiolabeled hormone, is measured using scintillation spectrophotometry. Known concentrations of unlabeled hormone, prepared in media or buffer, are subjected to the same experimental procedure in order to generate a standard curve. Quantification of the steroid level in the sample is ascertained by aligning values of the inhibition curve, generated using stable concentrations of radiolabeled hormone, with those of the standard curve. Each sample is measured in duplicate for the selected hormones and corrected for an extraction efficiency. Cross-reactivities of the hormones being tested with other steroid hormones are measured and reported.

Factors That Can Affect Sex Steroid Hormones

Circulating sex steroid levels are subject to normal

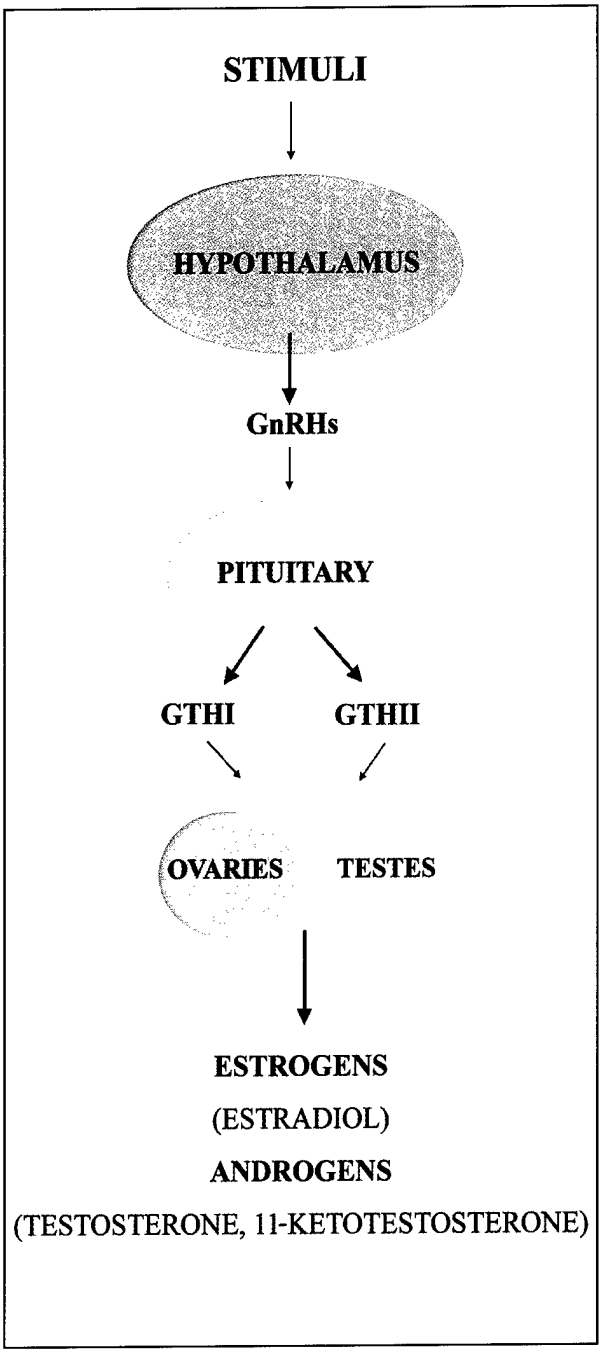


Figure 15. The reproductive hormone pathway in teleosts. GnRH = gonadotropin-releasing hormone, GTH-I and -II = gonadtropin hormones.

variation due to differences in sex, age, geographical location, species, and season (Goodbred et al. 1997; Denslow et al. 1998; Chang and Chen 1990; Barry et al. 1990; Down et al. 1990; Bromage et al. 1982; So et al. 1989). Several studies document the greatest variance in sex steroid concentrations during spawning, yet hormone levels from individuals at the same site have been shown to vary up to 30-fold during gonadal recrudescence (Chang and Chen 1990; Down et al. 1990; Folmar et al. 1996). There is also evidence that the stress of collecting, holding, and obtaining blood from fish may affect hormone concentrations (Jardine et al. 1996; McMaster et al. 1994; van den Heuvel et al. 1995; Barton and Iwama 1991; Magri et al. 1982), so conservative sampling protocols should be used.

In terms of xenobiotics, numerous studies demonstrate that exposure to a variety of structurally unrelated contaminants, including bleached kraft mill effluent (BKME) (McMaster et al. 1991; Munkittrick et al. 1991; Munkittrick et al. 1992; Munkittrick et al. 1994), agricultural pesticides (Goodbred et al. 1997; Gross et al. 1997; Singh et al. 1994; Singh and Singh 1987; Singh and Singh 1991), industrial chemicals (Sivarajah et al. 1978; Spies et al. 1996), and heavy metals (Allen-Gil et al. 1993; Thomas 1988), can lead to alterations in plasma sex steroid concentrations in a variety of fish species. For example, Grady et al. (1998) found that environmentally relevant concentrations of atrazine affected hormone concentrations in both male and female largemouth bass (*Micropterus salmoides*) (Fig. 16). There is substantial evidence to suggest that pollutants can alter sex steroid levels by interfering at multiple sites along the hypothalamo-pituitary-gonadal axis. Mukherjee et al. (1991) reported that phenol and sulfide, two components of BKME, disrupt steroidogenesis by inhibiting the conversion of cholesterol to sex steroids in the gonads. Heavy metals, on the other hand, may reduce sex steroid levels by stimulating the production of cortisol, which subsequently accelerates the metabolism of steroids in the liver (Hansson 1981). It is also possible that multiple endocrine pathways are affected simultaneously (Singh et al. 1994), especially if wildlife populations are exposed to combinations of chemicals. Consistent with this theory, van der Kraak et al. (1992) reported that in addition to increasing the metabolism of sex steroids in the liver, BKME interfered with the release of gonadotropins from the pituitary and suppressed steroid synthesis in the ovaries.

Value and Utility of Sex Steroid Hormones in the BEST Program

Current risk assessment strategies are generally designed to detect and characterize acutely hazardous chemicals that pose potential health risks such as cancer and birth defects. However, the effects of environmental stressors on the endocrine and reproductive systems are often subtle, making them difficult to

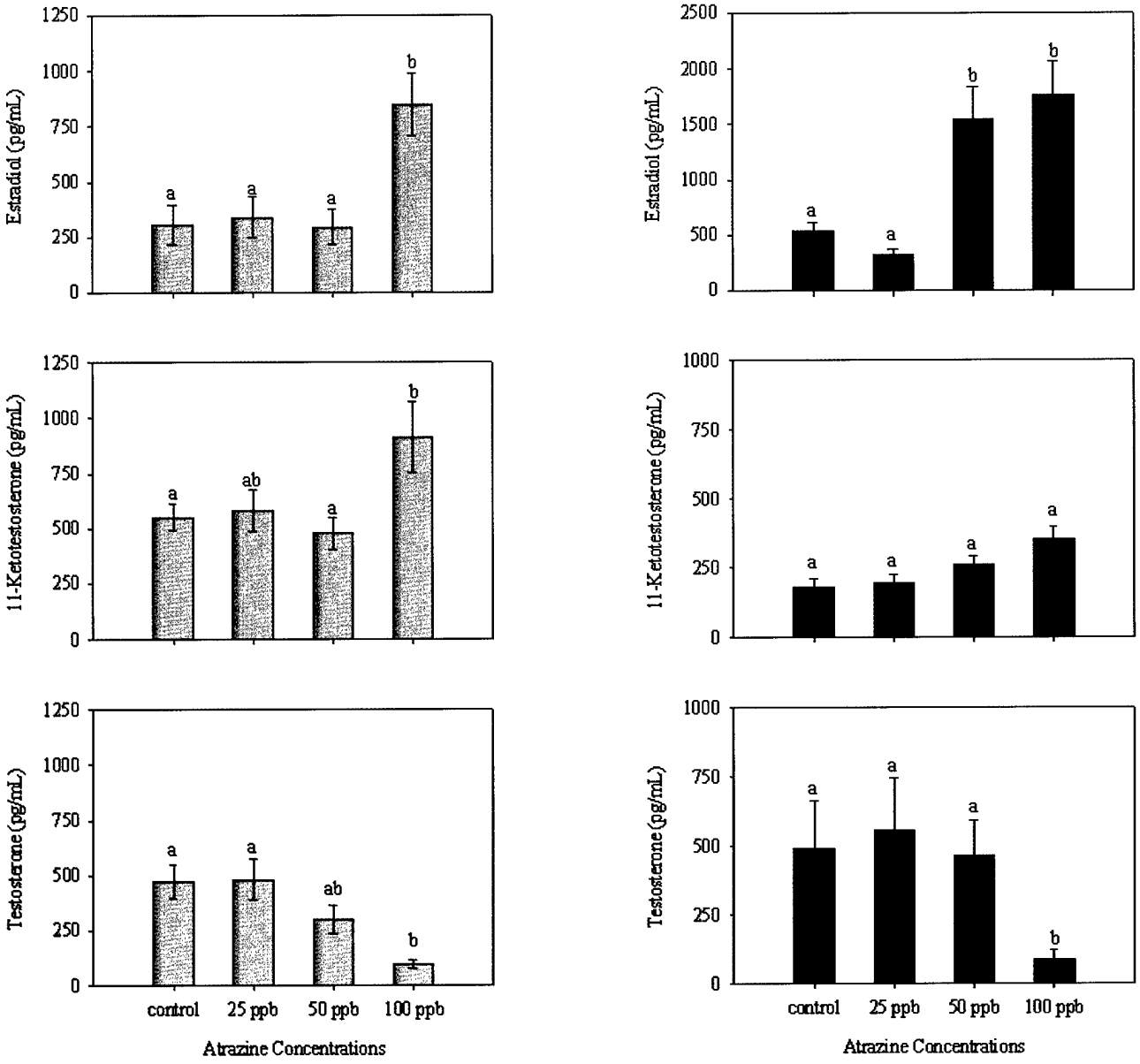


Figure 16. Mean plasma steroid concentrations for male (blue) and female (red) largemouth bass following a 20-d exposure to atrazine.

identify using traditional protocols. Measuring reproductive hormone levels provides one method for monitoring subtle physiological alterations that may lead to long-term, detrimental population effects. Reviews by several investigators correlate reduced sex steroid levels, in response to contaminant exposure, with reproductive impairment (Colborn and Clement 1992; Mayer et al. 1992), although the issue of determining cause-and-effect from sex steroid hormone studies remains somewhat controversial.

Although several caveats exist, measuring sex steroids is rapid and relatively inexpensive and can provide valuable biochemical and physiological information. With regard to natural variance, it is important to determine the base-line values and consider the environmental factors that may influence the reproductive physiology of the species being investigated. In general, sex steroids as biomarkers for reproductive status and health serve as convenient early indicators of stress and are most reliable when

field experiments are validated with laboratory experiments and species-specific physiology and environmental factors are considered. Other methods which examine reproductive capacity at different organizational levels (i.e., community, species, tissue, cellular, and subcellular) should also be employed to aid in interpretation of field data particularly since, with the exception of a few well-documented studies (Munkittrick et al. 1991; Sepulveda et al. 1998), information regarding ecological implications of hormonal modulations in fish exposed to harmful chemicals is limited.

VITELLOGENIN

Vitellogenesis usually occurs during the prespawning season in oviparous fish (Scott and Sumpter 1983; Lamba et al. 1983). GTH-I, released from the pituitary, stimulates the production of 17β-estradiol in the ovaries (reviewed by Redding and Patino 1993). High levels of circulating 17β-estradiol stimulate the liver to synthesize and release vitellogenin (vtg), a glycolipophosphoprotein egg yolk precursor (Fig. 17). Evidence for this estrogen-dependence comes from several studies in which high concentrations of estrogens have been reported prior to, or concurrent with, the onset of vitellogenesis in a number of fish including European flounder (*Platichthys flesus*) (Emmersen and Petersen 1976), stinging catfish (*Heteropneustes fossilis*) (Sundararaj et al. 1982), and chum salmon (*Oncorhynchus keta*) (Ueda et al. 1984). In addition, administering estradiol to males and immature (non-vitellogenic) females has been reported to induce vtg production in a number of studies (Emmersen and Petersen 1976; Campbell and Idler 1980; Korsgaard et al. 1983). After being released by the liver into the bloodstream, vtg is delivered to the ovaries where it is recognized by high-affinity receptors on the surface of the oocyte and subsequently internalized by receptor-mediated endocytosis (Fig. 17) (Specker and Sullivan 1994). In the final step, vtg is enzymatically cleaved within the oocyte to form the yolk proteins that serve to nourish the developing embryo. Depending on the species of fish and the experimental technique used, GTH-I, GTH-II, or both influence the uptake of vtg by the developing oocyte. In rainbow trout (*Oncorhynchus mykiss*), *in vivo* and *in vitro* methods have documented the involvement of GTH-I, but not GTH-II (Tyler 1991), whereas experiments using vitellogenic goldfish (*Carassius auratus*) reported enhanced vtg uptake following exposure to both gonadotropins (Rodriguez and Peter, personal communication).

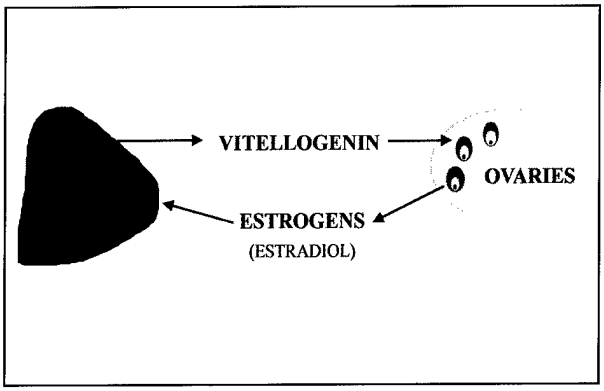


Figure 17. The role of the ovaries and liver in vitellogenesis.

Background

Vitellogenin is an estrogen-inducible protein that is normally synthesized by the liver of nonmammalian female vertebrates during oocyte development. The discovery that many structurally diverse chemicals [e.g., chlorobenzene and dichlorodiphenyltrichloroethane (DDT)] that are released into the environment possess estrogenic properties (Arcand-Hoy and Benson 1998; McLachlan 1993) has encouraged the development and utilization of bioassays that evaluate estrogenicity. However, the development of a universal assay has been challenging because some chemicals that bind the estrogen receptor do not elicit an estrogenic response and, conversely, some compounds evoke an estrogenic response without interacting with the receptor. This unpredictable structure-function relationship demands an assay based on the bioactivity of potential environmental estrogens. The fact that vtg synthesis is primarily regulated by circulating estrogens has made vtg an attractive indicator of potential estrogen action (Palmer and Palmer 1995; Palmer and Selcer 1996).

Performing the Vitellogenin Assay

Vitellogenin concentrations in plasma or serum can be determined using several different immunological methods. Sodium dodecylsulfate polyacrylamide gel electrophoresis and immunoblotting (Western analysis) can be used to detect vtg protein with an anti-vtg antibody, and relative protein concentrations can be quantified using densitometry. Alternatively, the enzyme-linked immunosorbent assay (ELISA) is less expensive and has become the screening method of choice for the BEST program (Folmar et al. 1996). Several vtg antibodies have been developed and well-

characterized (Heppell et al. 1995; Denslow et al. 1996), including the anti-carp vtg monoclonal antibody, Mab HL 1147 (2D3-3a9) (Folmar et al. 1996).

The following summarizes the ELISA protocol adapted from Folmar et al. (1996). Microtiter plates (96-well) are coated with purified anti-carp vtg monoclonal antibody and incubated overnight. The next day, plates are washed with Tris Buffered Saline/Tween 20 (TBST) solution and incubated with bovine serum albumin to block non-specific antibody binding. After thoroughly washing with TBST, plasma samples (diluted from 1:500 to 1:5000) are added in duplicate to the plates and incubated overnight. Standard curves are constructed by adding serial dilutions of purified vtg to male control plasma and processed according to the same method. The following day, the plates are washed with TBST and incubated with a rabbit anti-vtg polyclonal antibody for 2 h. The rabbit antibody binds to the vtg captured by the monoclonal antibody in the first step. The polyclonal antibody is in turn bound by a goat anti-rabbit IgG conjugated to alkaline phosphatase, which is applied to the wells and allowed to incubate for 2 h. After a final series of washes with TBST, *p*-nitrophenyl phosphate in carbonate buffer is added to each well and incubated for 30 min. The p-nitrophenyl phosphate serves as a substrate for the alkaline phosphatase and this reaction generates a yellow color that can be quantified by measuring the absorbance at 405 nm using an automated ELISA reader (Fig. 18). Vitellogenin concentrations are determined by subtracting values obtained from male control serum and comparing to standard curves.

Factors That Can Affect Vitellogenin

It is clear from the literature that the vitellogenic response is dependent on a number of intrinsic factors, including species, sex, and maturation/reproductive stage, as well as extrinsic factors, such as water temperature, season, and chemical composition of the aquatic environment (Wallaert and Babin 1994; Korsgaard et al. 1986). There is evidence for the regulation of vtg synthesis by circadian rhythms in catfish (Lamba et al. 1983), photoperiod in rainbow trout (Bromage et al. 1982), and winter sea temperatures in

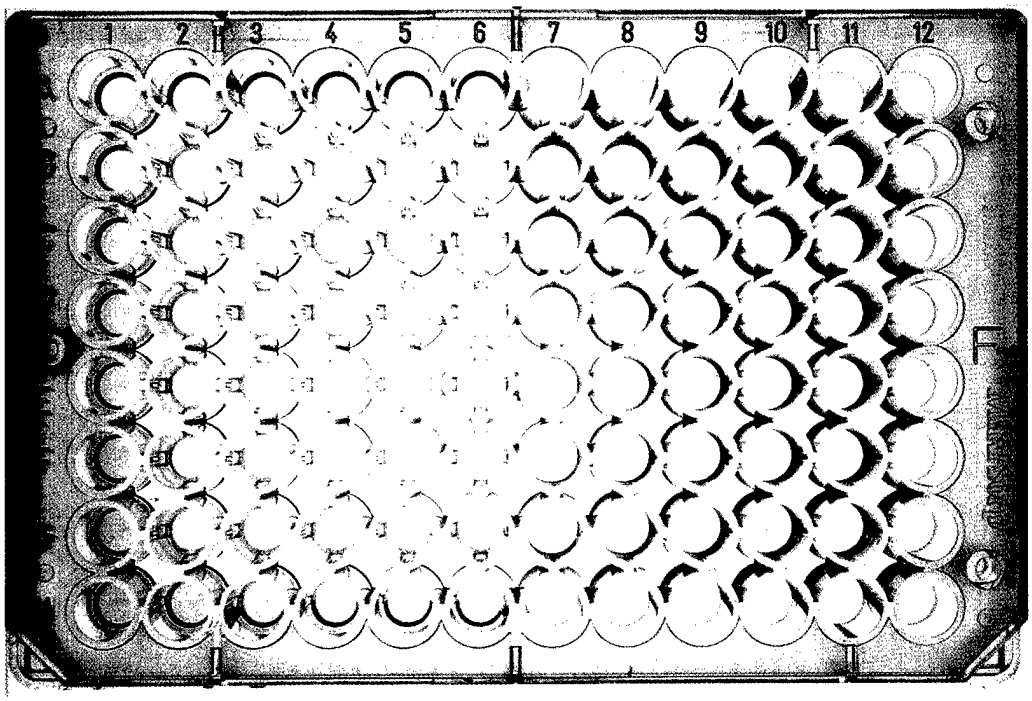


Figure 18. The standard curve in a vitellogenin assay (run in triplicate in columns 1-3, rows A-H) illustrating the gradation of color change. Columns 4-6 contain unknown plasma samples run in triplicate.

small-spotted catshark (*Scyliorhinus canicula*) (Craik 1978). In two independent studies, female Atlantic salmon (*Salmo salar*) and rainbow trout were monitored for vtg levels following exposure to cyanide. Plasma levels of vtg were reduced in rainbow trout (Ruby et al. 1986), whereas an increase in the plasma vtg concentration was reported in Atlantic salmon (So et al. 1987). The opposite response to the same environmental contaminant is not too surprising considering the differences in species, season, and exposure (concentration/duration). Interpretation becomes even more challenging when considering the natural variation in vtg synthesis and circulation that occurs between fish of the same species, sex, and geographic location. Sumpter and Jobling (1995) reported plasma vtg inductions ranging from 500-fold to over 50,000-fold in rainbow trout maintained in effluent from several neighboring sewage treatment sites.

Recent reports document enhanced vtg levels in both male and female fish exposed to various man-made and naturally occurring environmental agents. Some of the most compelling evidence for chemical-induced vtg production comes from field studies investigating the effects of sewage effluent and other chemicals on male and female fish residing downstream of treatment facilities (Bevans et al. 1996; Purdom et al. 1994; Sumpter and Jobling 1995). In one report, a 1000-fold increase in plasma vtg was observed in fish exposed for only three weeks to sewage effluent (Purdom et al. 1994). Corresponding laboratory experiments verified the induction of vtg following brief exposure to extremely low levels (0.1 ng/l) of 17β-ethinylestradiol, a potent estrogen and common constituent of sewage effluent (Purdom et al. 1994). Elevated plasma vtg levels have also been reported in response to a number of other environmental contaminants, including chlordecone, *o,p'*-DDT, and *o,p'*-dichlorodiphenylethylene (DDE) (Donohoe and Curtis 1996), dioxin (von der Decken et al. 1992), polluted harbor dredged spoil (Janssen et al. 1997), and the pesticide atrazine (Fig. 19) (Grady et al. 1998). Although the potentially harmful effects of environmental estrogens have received considerable attention, alterations in endocrine and reproductive functions have also been documented in fish exposed to a number of metals and inorganic molecules and compounds. Reductions in plasma vtg concentrations were reported in brook trout (*Salvelinus fontinalis*) exposed to various combinations of acid, aluminum, and calcium (Mount et al. 1988), winter flounder (*Pleuronectes americanus*) treated with cadmium (Pereira et al. 1993), and walking catfish (*Clarias batrachus*) injected with lead, zinc, and mercuric acetate (Panigrahi et al. 1990).

Value and Utility of Vitellogenin in the BEST Program

The measurement of vtg levels in female and male fish provides an additional biomarker for determining the stage of maturation, assessing reproductive health, and predicting the estrogenicity of various compounds; however, the researcher should be cautious, as a number of studies have reported considerable variability in both sexes. The literature also documents that the vitellogenic response is dependent on a number of intrinsic and extrinsic factors.

Although female vtg production can be affected by contaminants, plasma concentrations are so variable that it is difficult to determine the significance. Thus, vtg production by male fish became the focus of a number of investigations. Various studies led to the assumption that healthy males would not produce vtg and, therefore, the detection of plasma vtg in males would serve as a reliable biomarker of exposure to environmental estrogens (Palmer and

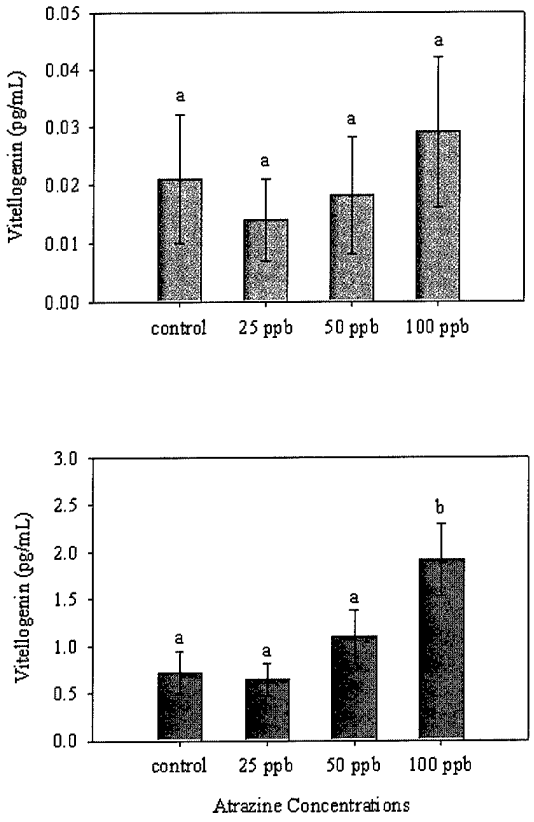


Figure 19. Mean plasma vitellogenin concentrations for male (blue) and female (red) largemouth bass following a 20-d exposure to atrazine.

Selcer 1996; Heppell et al. 1995; Sumpter and Jobling 1995). According to the literature, most males do not produce vtg in measurable quantities; nevertheless, vtg receptors have been detected in testes, muscle, and spermatocytes (Bidwell and Carlson 1995; Tao et al. 1996). Males treated with estrogen can produce significant concentrations of plasma vtg (Bromage and Cumaranatunga 1988; Christiansen et al. 1998), yet studies have also detected various levels of plasma vtg in untreated male fish from reference sites (Goodbred et al. 1997; Denslow et al. 1998). Given that the biological significance of vtg in males is currently unknown, correlating vtg production with endocrine disruption or feminization is risky in the absence of data corroborating such conditions (e.g., sex steroid measures, histopathology). Detrimental effects on wild populations cannot currently be ascribed to the unnatural or untimely production of vtg in adult males. Evidence of injury from vitellogenesis has only been seen in juvenile male rainbow trout that suffered renal problems, and ultimately death, after vitellogenesis was induced by pharmacological doses of 17β-estradiol (Herman and Kincaid 1988).

The development of a universal assay for detecting vtg by immunological methods has been complicated by the fact that the primary amino acid sequence is not highly conserved among species of fish (Campbell and Idler 1980; So et al. 1985; Benfey et al 1989; Lee et al. 1992). Nevertheless, monoclonal antibodies have been developed that cross-react with a wide range of species. For instance the monoclonal antibody, mAb 2D8 (Heppell et al. 1995) showed wide taxonomic specificity and cross-reacted with vtg from all species tested. But, because it was an IgM and of relatively low affinity, its usefulness for an assay was limited. Other monoclonal antibodies of the IgG class have been prepared that are of high affinity and very specific for vtg (Denslow et al. 1996; Denslow et al. 1997). These monoclonal antibodies are not "universal" but they do cross-react with vtg of fishes from diverse families, making them useful for evaluating vtg induction in the wild. In summary, vtg assays are sensitive and reasonably inexpensive; however, interpretation of findings, especially when less than 25% in a population have low amounts (<10 μg/ml), remains somewhat challenging. As is true of many emerging biomarkers, a better understanding of factors that either influence or inhibit vtg production in both males and females would improve the utility of vtg as an indicator of chemical exposure and reproductive health.

Gonado-Somatic Index (GSI) And Gonadal Histopathology (Including Gonadal Staging)

The GSI and gonadal histopathology fall into a category of indicators that provide structural, rather than functional, information about gonadal health and maturational stage. The GSI is one of several organosomatic indices, including the HSI and SSI, which establishes a ponderal relationship between the organ and the entire body. There is substantial evidence that most animal species undergo reproductive cycling and, frequently, dramatic variation in gonadal size is observed throughout the cycle (de Vlaming et al. 1981). Consequently, calculating gonadal weight as a percentage of body weight has routinely been used to determine reproductive maturity, as well as assess gonadal changes in response to environmental dynamics (e.g., seasonal changes) or exogenous stresses (e.g., contaminant exposure).

Gonadal histopathology is often utilized alone, or in conjunction with the GSI, to confirm gonadal phenotype, determine the state of sexual development, and investigate reproductive impairment. Although gonadal histopathology is routinely used to detect higher level responses expressed as morphological abnormalities, such as the presence of ovotestes (Fig. 20) or multinuclear ova, this method is capable of providing information at multiple levels of biological organization (i.e., distribution of molecules; distribution, number, volume, morphology of organelles, cells, and organs). Observed alterations in cells and tissues are often reflective of previous biochemical and physiological modifications.

Background

The utilization of the GSI as a reproductive biomarker was first reported in 1927 in a study describing the yearly variations of female yellow perch (*Perca flavescens*) ovaries (Meien 1927). Years later, Nikolsky (1963) endorsed this method on the premise that "...the effects of fish size on gonadal weight are eliminated by expressing gonadal weight as a percentage of body weight." There is significant evidence that exposure to various environmental pollutants can result in gonadal alterations such as a decreased GSI, morphological changes, or both.

Measuring the GSI and Performing Gonadal Histopathology

Measuring the GSI is a convenient and frequently

employed method that demands little time and few instruments. The GSI is calculated as (gonad weight/body weight) * 100. The only requirement is that the measurements be made on live or freshly killed specimens to avoid weight fluctuations induced by storage conditions (i.e., moisture loss/gain). Evaluating the condition of the gonads by histopathology is more labor intensive and requires experienced personnel. Briefly, isolated gonads are fixed with Bouins' or Smith's solution, a glutaraldehyde-paraformaldehyde solution, or some other preservative (e.g., NoTox or 10% neutral buffered formalin). Dehydration of the samples is performed by transferring them through a series of graded ethanol solutions (50-99%). Testes are cut longitudinally and ovaries are cut transversely before embedding in paraffin and thin-sectioning (0.5 μm). Prior to evaluation by light microscopy, the samples are treated with one or more staining compounds, such as hematoxylin, which stains nuclear elements, and eosin, which labels general cytoplasmic and intracellular materials.

To evaluate gonadal stage several classification schemes based on histological examination have been described and applied to the evaluation of gonadal development and the seasonal activity changes that occur in fish. The classification system adopted by Goodbred et al. (1997) describes four maturational stages for females and males. For females, stage 0 describes inactive ovaries containing primarily perinucleolar (primary or secondary) oocytes with no developing ova. The previtellogenic stage, or stage 1, contains a mixture of perinucleolar and cortical alveolar oocytes. Slightly to moderately enlarged ova containing vacuoles or lipid droplets, but few or no vitelline granules, are representative of this stage. Ovaries classified as early vitellogenic (stage 2) contain oocytes of various sizes and development. Few (or no) fully developed ova exist, although moderate numbers of vitelline granules may be present. The vitelline granules are storage compartments composed of lipid-bound vitellogenin fragments. Ovaries in the final stage of sexual development, classified as late vitellogenic (stage 3), contain ooyctes approaching maximum size that consist of numerous densely packed vitelline granules. Although stage 3 is typical of fish approaching spawning, stage 1, 2, and 3 are all representative of sexually mature female fish. Wallace and Selman's version of teleostian oocyte maturation is similar to that above, although they describe six stages of development: the chromatin-nucleolus phase, perinuclear phase, cortical alveoli phase, vitellogenic phase, maturation phase, and ovulation phase (Wallace and Selman 1990).

The classification system adopted by the BEST Program for common carp (*Cyprinus carpio*) and black basses is similar to that of Wallace and Selman (1990) in that it recognizes six developmental stages for females and four maturational stages for males. The stages are based on the size and developmental status of the oocytes (ovaries) and spermatozoa (testes) contained within the reproductive organs (Table 6). In females, stage 0 (immature) ovaries contain pre-vitellogenic oocytes in the chromatin-nucleolus and early perinucleolus stages. Stage 1 ovaries contain oocytes in early development; more than 90% are pre-vitellogenic and the remainder are early to mid-vitellogenic. The early vitellogenic oocytes are in the late perinucleolus through cortical alveolar stages and are slightly larger than the pre-vitellogenic oocytes. The mid-vitellogenic oocytes contain yolk vesicles, which appear as globules around the periphery of the cytoplasm. The chorion is uniformly apparent and the oocyte is larger. Stage 2 ovaries contain oocytes in mid development. In this stage the majority of developing follicles are early to mid-vitellogenic. In late development, or Stage 3, the majority of developing follicles are late vitellogenic, which is characterized by increased oocyte diameter and chorion thickness and yolk globules distributed throughout the cytoplasm. Stage 4 is the late development/hydrated stage in which the majority of developing follicles are late vitellogenic and significantly larger than stage 3 follicles. Stage 5, or the post-ovulatory stage, is characterized by the presence of spent follicles marked by remnants of the theca externa and granulosa. Although stage 3 is typical of fish approaching spawning, stages 1, 2, and 3 are all representative of sexually mature female fish. It should be noted that the classification schemes for females described here are relatively general, and all stages may not be evident in females of every species.

For males, the gonadal stages recognized in fish collected by the BEST program (Table 6) are for all practical purposes identical to those used by Goodbred et al. (1997). The stages are based on the maturity of the predominant stage of spermatogenesis (Nagahama 1983). Stage 0 (immature) in males is recognized by the absence of spermatogenic activity in the germinal epithelium and the presence of primarily spermatocytes. No spermatozoa are present in the tubules. Testes in early spermatogenesis (stage 1) are characterized by mostly thin germinal epithelium and the presence of primarily immature cells (spermatocytes to spermatids); however, some spermatozoa are also present. In mid-spermatogenesis (stage 2) the germinal epithelium is moderately thick and some proliferation and maturation of sperm can be observed; spermatocytes, spermatids, and spermato-

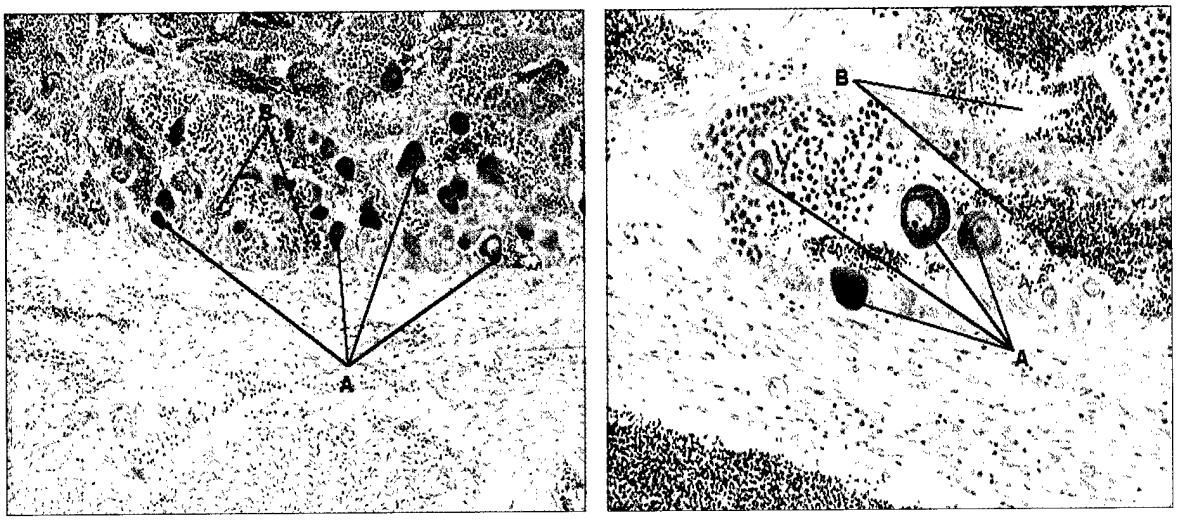


Figure 20. Histologic appearance of intersex fish. Left panel: Low magnification (165x) showing oocyte (A) development alongside sperm (B) within maturing testicular tissue. Right panel: A higher magnifcation view (412x).

zoa are present in roughly equal proportion. In late spermatogenesis (stage 3) the germinal epithelium is thick. Although all cell types are represented, spermatozoa predominate in stage 3. Stages 1 through 3 are characteristic of sexually mature fish, with the least activity occurring in off-season (stage 1) and the most activity taking place immediately prior to and during the spawning season (stage 3).

Factors That Can Affect the GSI and Gonadal Histopathology

As noted above, both the GSI and gonadal histopathology are affected by season, which controls reproductive cycling. The effect of age on the GSI and gonadal histopathology is reviewed in a recent article by Patnaik et al. (1994). Gender influences the GSI with males experiencing less gonadal weight gain during recrudescence than females. Males also have less well-defined stages of maturation (Kime 1995). Variability in these parameters also exists among species. For example, goldfish and other small cyprinids are fractional spawners (Delahunty and de Vlaming 1980), depositing eggs during isolated episodes over the course of several months (Yamazaki 1962). Unlike species that spawn once a year, or once during a lifecycle, the GSI and gonadal structure change more frequently as the fractional spawners undergo repeated oocyte maturation and release.

Pollutants may also cause alterations in these two indicators. A reduction in the GSI and impaired gonadal development (growth and structural pathologies) have been reported in response to environmentally relevant doses of dietary mercury in juvenile walleye (*Stizostedion vitreum*) (Friedmann et al. 1996), organophosphate insecticides in female striped catfish (*Mystus vittatus*) (Choudhury et al. 1993), and metacid-50 and carbaryl in climbing perch (*Anabas testudineus*) (Haider and Upadhyaya 1985). In a field study, fathead minnows (*Pimephales promelas*) were subjected to acidic water conditions and oocyte atresia was detected histologically (McCormick et al. 1989). Oocyte atresia, as defined by an involution or resorption of unfertilized eggs by the ovaries, is a normal physiological event in all fish, but it has become a pathological condition noted in fish after exposure to certain environmental contaminants (Fig. 21) (Johnson et al. 1988; Cross and Hose 1988; Cross and Hose 1989; Kirubagaran and Joy 1988). The ability to detect increased degeneration or necrosis of developing oocytes by histological examination has inspired the use of oocyte atresia as a biomarker of reproductive impairment.

Value and Utility of the GSI and Gonadal Histopathology in the BEST Program

Over the years, the accuracy and reliability of the GSI measurement have been scrutinized by some, due to the variability observed between relative ovarian weights even within the same or related species (de Vlaming et al. 1981) and the inability of some field studies to correlate tissue levels of contaminants or plasma sex steroid levels with the GSI (Johnson et al. 1994; Monosson et al. 1994; Sepulveda et al. 1998). Others suggest that a correlation between all levels of organization is not necessary and that a negative response from one marker (e.g., the GSI) does not

negate or lessen the significance of meaningful data collected from a nonequivalent bioassay (e.g., sex steroid analysis) (Huggett et al. 1992).

Despite conflicting opinions regarding its reliability, the GSI is easy and inexpensive to measure, and has remained a commonly used criterion for evaluating the reproductive status and health of fish. As with the evaluation of sex steroid hormones and vitellogenin, interpretations of GSI measurements rely on a thorough understanding of the natural variability between fish of the same age, sex, and species, as well as the environmental influences, behavioral patterns, and reproductive strategies that may complicate or confound data. For example, in one study it was shown that winter flounder remain offshore during most of early vitellogenesis and move into contaminated estuaries prior to spawning, whereas English sole (*Parophrys vetulus*) reside in contaminated estuaries during vitellogenesis and move offshore to spawn. The migratory behaviors of the two species influences the duration and timing of exposure to pollutants and, therefore, can have an indirect impact on gonadal recrudescence (Johnson et al. 1994).

Many biomarkers vary seasonally with the reproductive cycle. To interpret these biomarkers, one must know the reproductive status of each fish, which can be achieved by determining its gonadal stage or condition histologically. Histological examination alone is also a valuable technique that has been used to evaluate reproductive status, determine sex, and assess the gonadal health of male and female fish. In addition to the advantages described in the intro-

Table 6. Histological features of gonadal stages as defined for use in the BEST program. Adapted from Treasurer and Holiday, 1981, Nagahama 1983, Rodriquez et al. 1995 and Goodbred et al. 1997.

Stage	Testis characteristics (male)	Ovary characteristics (female)
0	Undeveloped (immature): Little or no spermatogenic activity in germinal epithelium; immature states of spermatogenesis (largely spermatocytes); no spermatozoa observed.	Undeveloped: Pre-vitellogenic oocytes observed exclusively; oocyte diamter <250 µm: cytoplasm stains basophilic with H&E.
1	Early spermatogenic: Mostly thin germinal epithelium with scattered spermatogenic activity; spermatocytes to spermatids predominate; few spermatozoa observed.	Early development: >90% pre-vitellogenic, remaining oocytes early to mid-vitellogenic; oocytes slightly larger (up to 300 µm); late perinucleolus through cortical alveolar stages.
2	Mid-spermatogenic: Germinal epithelia are of moderate thickness; moderate proliferation and maturation of spermatozoa and equal mix of spermatocytes, spermatids and spermatozoa present.	Mid-development: Majority of observed follicles are early and mid-vitellogenic; oocytes larger, 300-600 µm diameter, and containing peripheral yolk vesicles; globular and uniformly thick chorion (5-10 µm in black basses, 10-20 µm in common carp); cytoplasm is basophillic, yolk globules eosinophillic.
3	Late spermatogenic: Thick germinal spithelium; diffuse regions of proliferation and maturation of spermatozoa; all stages of development are represented, but spermatozoa predominate.	Late development: Majority of developing follicles are late vitellogenic; oocyte diameter is 600-1000 µm; eosinophillic yolk globules distributed throughout the cytoplasm; chorion thickness is 10-30 µm in black basses, 40-50 µm in common carp.
4	N/A	Late development/hydrated: Majority of developing follicles are late vitellogenic; follicles are much larger (>1000 µm).
5	N/A	Post-ovlulatory: Spent follicles, remnants of the theca externa and granulosa.

duction to this section, histopathology can often provide information regarding the magnitude, or occasionally the source, of toxic impairment. In addition, this method may be used to study alterations in animals that are too small for standard biochemical analyses. For accurate interpretation of histological data, the investigator must have extensive knowledge of the gross and microscopic anatomy of the specimen under investigation and must be aware of normal variations in the anatomy of the species being examined, as well as differences that may occur due to gender, age, and seasonal variations. In addition, the investigator must be able to differentiate between lesions of different origins, including exposure to toxic chemicals, infectious diseases, congenital anomalies, and stress from handling. Using control animals in experimental studies will help distinguish between normal morphology and lesions derived from the experimental treatment. Although the subjectivity of the examiner can also lead to variance, microcomputers and associated software programs now exist that allow for the quantitative analysis of morphological data. To date, oocyte atresia is the only histological biomarker that has been sufficiently validated; that is, lesions in laboratory studies have been correlated to chemical exposure, and these same lesions detected in fish from contaminated sites (Hinton et al. 1992). Also, a potential correlation has been found between oocyte atresia and reproductive success (McCormick et al. 1989). A number of other lesions (e.g., testicular atrophy, sperm reduction) have potential as histopathological biomarkers; however, field confirmation of laboratory results is needed before these indicators can be used with confidence.

Summary

At the biochemical level, the sex steroids respond quickly to both intrinsic (physiological) and extrinsic (environmental) stimuli, making these indicators rapid and relatively easy to measure. They are often the first detectable responses to an environmental change or stress and can serve as indicators of both exposure and effect. Furthermore, effects at the biochemical level are highly sensitive and often underlie changes observed at more complex levels of organization and, thus, may be predictive of perturbations at the organ or organism level. Although the high sensitivity of the sex steroids can complicate data interpretation when considering the number of factors (e.g., temperature, photoperoid, handling and drawing blood) that can affect circulating levels, many of these problems can be overcome in both laboratory and field studies.

Vitellogenin is another highly sensitive biomarker with the potential to provide information regarding exposure and effect at the biochemical level. It was originally hypothesized that vtg production in males was indicative of estrogen exposure and would serve as an effective biomarker of endocrine disruption. Although this may be the case, recent studies suggest that even healthy males may produce low levels of vtg, and there is currently no evidence that trace concentrations affect either general health or reproduction. Vitellogenin has proven to be a valuable biomarker in carefully controlled laboratory experiments as well as isolated field studies. However, the successful application of vtg as a biomarker for males in large-scale field studies is contingent on understanding the normal patterns of vtg production in males. Until then, vtg may be a more reli-

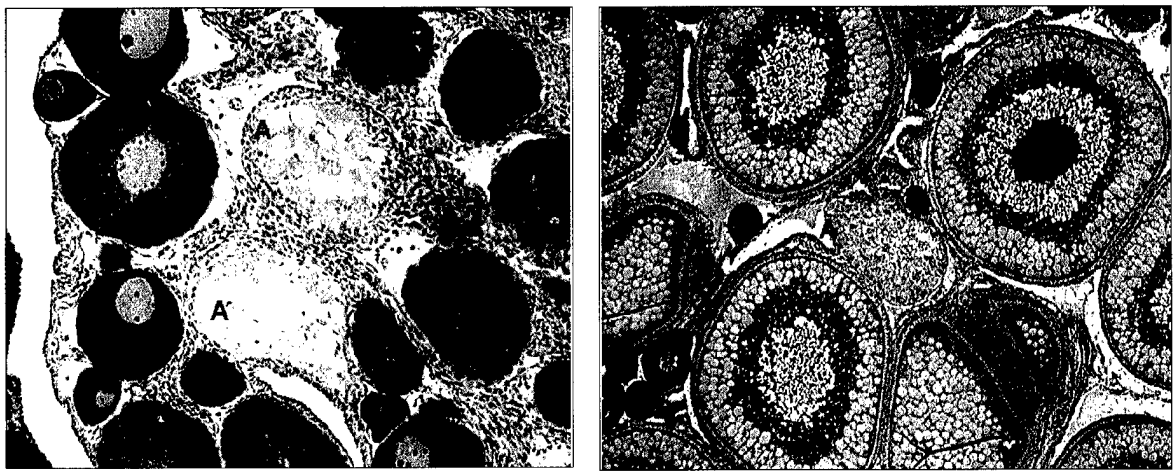


Figure 21. Atretic eggs (A) present in ovaries at two different stages of development. Left panel, 412x, right panel, 165x.

able biomarker in females. For instance, in males, vtg has no known function, whereas in females, it is a critical component of the developing oocyte. Therefore, identifying females with low or undetectable vtg may be as important as detecting males with vtg. Since all female oviparous fish produce vtg, usually in the prespawning period, a lack of vtg may indicate serious reproductive problems.

The GSI and histological examination provide structural information concerning the gonads and appear to be advantageous methods for identifying effects of long-term contaminant exposure. The GSI is easy and inexpensive, although definitely crude in comparison to the more informative, yet more labor-intensive, histological exam. Both techniques require knowledge of the variations due to age, species, and season, and histology requires extensive knowledge of the gross and microscopic anatomy of the specimen under investigation. Although it requires a qualified pathologist, information gained from gonadal histopathology, such as sex and reproductive stage, is essential for interpreting many of the other biomarker responses. In addition, histopathology can provide unique information from the visual examination of gonadal tissue, including the assessment of oocyte atresia and detection of ovotestes.

In addition to the reproductive biomarkers discussed in this review, information regarding a variety of other parameters including fertility, clutch size, hatchability, and sexual behavior are necessary for a complete understanding of the reproductive mechanisms in healthy and compromised wildlife populations, which cannot be assessed at the geographic scale represented by the BEST program. Currently, the optimal approach for studying the reproductive system at this scale involves using a suite of biomarkers to gain information at different levels of organization. Each of the above indicators has advantages and disadvantages, but analyzing them together may help to overcome the individual weaknesses of any one test. Individually, the reproductive biomarkers can provide only limited information regarding the effects of environmental stressors. However, when used together, the biomarkers described here can provide a more holistic account of an animal's reproductive health, allowing investigators to evaluate whether a species or population is at risk.

STABLE NITROGEN ISOTOPE RATIO ($\delta^{15}N$)

Christopher J. Schmitt

The ratio ($\delta^{15}N$) of the abundance of naturally occurring stable (non-radioactive) isotopes of nitrogen (^{15}N: ^{14}N) in organisms reflects both nitrogen sources to the ecosystem and trophic relationships within the ecosystem. $\delta^{15}N$ increases predictably upward through aquatic food chains at the rate of about 3-4% per trophic level (DeNiro and Epstein 1978; DeNiro and Epstein 1981; Minagawa and Wada 1984; Peterson and Fry 1987; Kling et al. 1992; Cabana et al. 1994; Michener and Schell 1994; Hobson and Welch 1995; Vander Zanden and Rasmussen 1996; Vander Zanden and Rasmussen 1999; Vander Zanden et al. 1997; Vander Zanden et al. 1998) (Fig. 22). In addition, nitrogen sources (i.e., fertilizers vs. animal wastes) commonly differ in isotopic composition and can be differentiated on the basis of their isotopic signature (e.g., Heaton 1986; Kendall 1998).

Background

Under ideal circumstances, stable nitrogen isotopes offer a direct means of source identification because the two major sources of nitrate in many agricultural areas, fertilizer and manure, generally have isotopically distinct $\delta^{15}N$ values. Hence, under favorable condi-

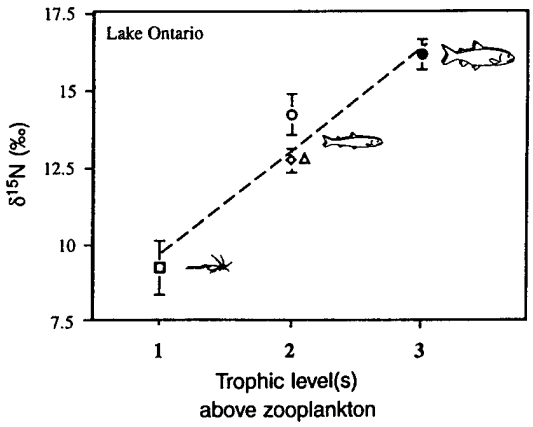


Figure 22. Mean (±s.e.) $\delta^{15}N$ for components of the Lake Ontario pelagic food chain. *Myis relicta*, pelagic forage fish (smelt, alewife, sculpin) and lake trout are represented by, respectively, square, circle, diamond, triangle, and filled circle (From Cabana and Rasmussen 1996; reproduced by permission).

tions, the relative contributions of these two sources to groundwater or surface water can be estimated by simple mass balance. Soil-derived nitrate and fertilizer nitrate commonly have overlapping $\delta^{15}N$ values, preventing their separation using $\delta^{15}N$ alone.

Animal waste (including sewage) almost always has a higher $\delta^{15}N$ than other nitrogen sources [e.g., fertilizers and atmospheric deposition (Heaton 1986)]. Animal-derived nitrogen in biological systems is indicated by a relatively high $\delta^{15}N$ in organisms at all trophic levels as sewage nutrients enter and pass through the food chain. Because of this property, $\delta^{15}N$ has been used to trace sewage-impacted groundwater inputs into a coral reef in Barbados (Cabana unpub. data). In this latter study, the indicator organisms were large sedentary invertebrates (mussels, chitons) and non-migratory fishes - guilds of organisms chosen for monitoring aquatic habitats by the BEST program (BEST 1996).

Measuring Stable Nitrogen Isotopes

^{14}N and ^{15}N (and thus $\delta^{15}N$) can be readily measured by mass spectrometry. Samples of dried, homogenized muscle or whole fish (about 2-3 mg dry weight) are pulverized and introduced in small tin boats prior to flash combustion, purification, and determination of isotopic ratios by mass spectrometry (Cabana and Rasmussen 1996).

Factors That Can Affect $\delta^{15}N$

An early attempt to use natural $\delta^{15}N$ values to determine sources of nitrate in surface waters (Kohl et al. 1971) received a highly critical response (Hauck et al. 1972). This was partly because the use of the $\delta^{15}N$ values of fertilizer and animal waste to trace their relative contributions to groundwater is complicated by several reactions (e.g., ammonia volatilization, nitrification, denitrification, ion exchange, and plant uptake) taking place within the hydrologic system that can significantly modify the $\delta^{15}N$ values. Moreover, mixing of point and non-point sources along shallow flow paths makes determination of sources and extent of denitrification very difficult. Because of these problems, attempts to use $\delta^{15}N$ for tracing the source and fate of nitrate in ground waters and surface waters often have only limited success, despite the moderately good separation of $\delta^{15}N$ values.

In addition to the problems cited above, other factors may also contribute to variability and uncertainty in the use of $\delta^{15}N$ as a nitrogen source tracer (Battaglin et al. 1997; Kendall 1998). These include variation in the isotopic composition of different sources of N as well as post-depositional recycling N (e.g., volatilization of ammonia, nitrification, denitrification, assimilation, etc.). In addition, there is the obvious confounding effect caused by trophic dynamic differences among ecosystems. Cabana and Rasmussen (1996) showed that in Ontario (Canada) lakes, $\delta^{15}N$ in organisms increases with trophic position and is heavily influenced by land use, in particular by inputs of sewage (a correlate of human population density).

$\delta^{15}N$ may also change over the life of an organism, reflecting changes in feeding habits and trophic position. In smallmouth bass (*Micropterus dolomieu*), for example, $\delta^{15}N$ in eggs were similar to adult levels, reflecting the trophic position of the parent. Levels decreased steadily from hatching through swim-up and metamorphosis, then began increasing after metamorphosis as the fish began to feed on progressively larger organisms (Vander Zanden et al. 1998).

Value and Utility of $\delta^{15}N$ in the BEST Program

Because it indicates the trophic position of the organisms being collected, $\delta^{15}N$ can be used as a corollary variable for interpreting otherwise unexplainable differences in organism concentrations of biomagnifying contaminants (e.g., mercury; polychlorinated biphenyls; Fig. 23). This method therefore allows one to account for site-specific differences in trophic position within and among species [i.e., between

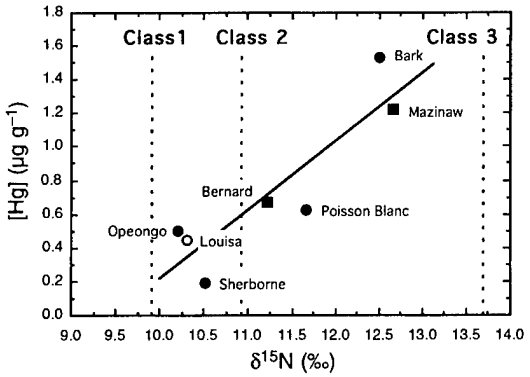


Figure 23. Relationship between mean mercury concentration (wet weight) and mean $\delta^{15}N$ in lake trout in seven Ontario and Quebec lakes (From Cabana and Rasmussen 1996; reproduced by permission).

smallmouth bass and largemouth bass (*Micropterus salmoides*) at the same and different sites] and to reduce the confounding effect of collecting different taxa at stations (Kiriluk et al. 1995; Kidd et al. 1995). Many studies (Purdom et al. 1994; Goodbred et al. 1997; Folmar et al. 1996; Bevans et al. 1996) have also shown that endocrine-modulating substances (e.g., alkylphenols, pharmaceuticals, phytoestrogens) are released from sewage treatment plants. In addition, recent evidence (Halling-Sørensen et al. 1998) suggests that pharmaceuticals are also released to surface waters from confined animal feeding operations (i.e., feedlots and pork and poultry production facilities). By serving as a means of differentiating animal waste (including human sewage) inputs from fertilizer inputs, $\delta^{15}N$ may be useful for interpreting the results of reproductive biomarkers. $\delta^{15}N$ may also suggest causal mechanisms behind certain fish kills and disease outbreaks; animal wastes are believed to be involved in outbreaks of *Pfisteria piscicida* and other toxic dinoflagellates responsible for recent, massive fish kills in Mid-Atlantic and Southeastern estuaries (Burkholder et al. 1995). $\delta^{15}N$ also represents a surrogate method for identifying and evaluating nutrient sources, a class of contaminants of great current concern in the Mississippi River basin because agriculturally derived nitrogen is believed to be responsible for the eutrophication of the Gulf of Mexico and the occurrence of anoxic zones (Rabalais et al. 1996; Battaglin et al. 1997). Excess nutrients are also often involved in nuisance blooms of cyanobacteria (*Nodularia, Anabaena, Aphanizomenon, Microcystis*) and dinoflagellates (e.g., *Protogonyaulax temorensis*) that produce potent hepatotoxins and neurotoxins known to affect fish and wildlife (Gosselin et al. 1989; Kotak et al. 1993; Carmichael 1994). Elevated $\delta^{15}N$ in aquatic organisms therefore indicates animal-derived nitrogen. The confounding effects of trophic position must be understood and accounted for, but the measurement of $\delta^{15}N$ represents a potential tool for identifying pollution impacts on water quality and for explaining temporal and geographic variability in monitoring studies.

APPENDIX I: DEVELOPMENT OF THE BEST PROGRAM

Christopher J. Schmitt

The National Contaminant Biomonitoring Program

The publication *of Silent Spring* (Carson 1962) called public attention to the environmental threat represented by dichlorodiphenyltrichloroethane (DDT) and, later, other persistent toxins that have the potential to bioaccumulate and harm wildlife. Member agencies of the Federal Committee on Pesticides responded by implementing the National Pesticide Monitoring Program (NPMP) to obtain information on concentrations and distributions of these substances (Geary 1967). Participating agencies designed and operated monitoring networks based on those segments of the environment for which each had responsibility. The U.S. Fish and Wildlife Service (FWS) participated in the NPMP by collecting and analyzing freshwater fish and European starlings (*Sturnus vulgaris*) and by analyzing the wings of hunter-killed ducks (Johnson et al. 1967) under its National Contaminant Biomonitoring Program (NCBP). Although the environmental matrices collected and analyzed differed among agencies, all components of the program were designed with the objective of monitoring temporal and geographic trends in the concentrations of specific contaminants, including DDT and mercury. Among the analytes added later were other organochlorine pesticides, such as dieldrin; polychlorinated biphenyls (PCBs) and other toxic industrial chemicals; and inorganic contaminants, including arsenic and selenium.

The NCBP, along with the NPMP-derived networks of other agencies, was highly successful in achieving the primary program objective of documenting the distribution of the monitored contaminants in space and time, thereby providing positive feedback to the regulatory process. Collectively, the networks had demonstrated that by the 1980s restrictions on the use and discharge of accumulative toxins had generally reduced concentrations of organochlorine pesticides, PCBs, and some inorganic contaminants in biota (Prouty and Bunck 1986; Bunck et al. 1987; Wade et al. 1988; Schmitt and Brumbaugh 1990; Schmitt et al. 1990; Sericano et al. 1990a, b; O'Connor 1991; Turgeon and Robertson 1995; Schmitt and Bunck 1995; Schmitt et al. 1999), but that there were still areas of the U.S. where these contaminants could represent a threat to fish and wildlife.

Fish Sampling Under the NPMP and NCBP

The fish component of the NPMP-NCBP was designed to monitor spatial and temporal trends in the concentrations of bioaccumulable contaminants over large expanses of the U.S. Fish were collected periodically from a national network of stations located at or near key points, such as at the confluences of major tributaries or below dams, in the larger rivers of the 50 states and in the open waters of the Great Lakes (Fig. A-1). Station locations were fixed only to the extent that the general area of the water body to be sampled was identified. Because there are no truly ubiquitous fishes that can be sampled throughout the United States, cooperators were instructed to collect representative species from two guilds—bottom-dwelling/feeding and piscivorous (Johnson et al. 1967). Species were selected from a list of preferred taxa stratified by aquatic habitat type — -cold-, cool-, or warm-water. Samples comprised 3-5 adult fish of a single species. Because of the recognized need to evaluate the transfer of organochlorine chemicals, mercury, and other bioaccumulable contaminants to higher trophic levels (i.e., piscivorous birds and mammals), fish were analyzed whole; for reasons of economy, the 3-5 individual fish comprising a sample were composited for chemical analysis. The overriding assumption was that periodic collections and analyses of whole fish from key locales would integrate large expanses of time and space with respect to the concentrations of the measured contaminants. Results could then be generalized to taxa other than those collected and used to assess the risk to piscivorous fishes and wildlife represented by the contaminants measured in the fish.

Shortcomings of the NCBP Fish Network

By the mid-1980's the FWS perceived that the NCBP was no longer meeting its growing need for contaminant-related information. Although the program provided data and information on the general distribution and concentrations of contaminants in the environment, the information was not focused on the waters, lands, and species of greatest concern to the FWS. The information produced by the program was generally perceived to be scientifically authoritative and academically interesting, but only marginally relevant to policy-makers due to the geographic, programmatic, taxonomic, and chemical shortcomings outlined here.

Geographic, Programmatic, and Taxonomic Shortcomings

As a result of government-wide reorganizations in the early 1970's, the regulation and management of hazardous substances in the environment became the responsibility of the newly created U.S.

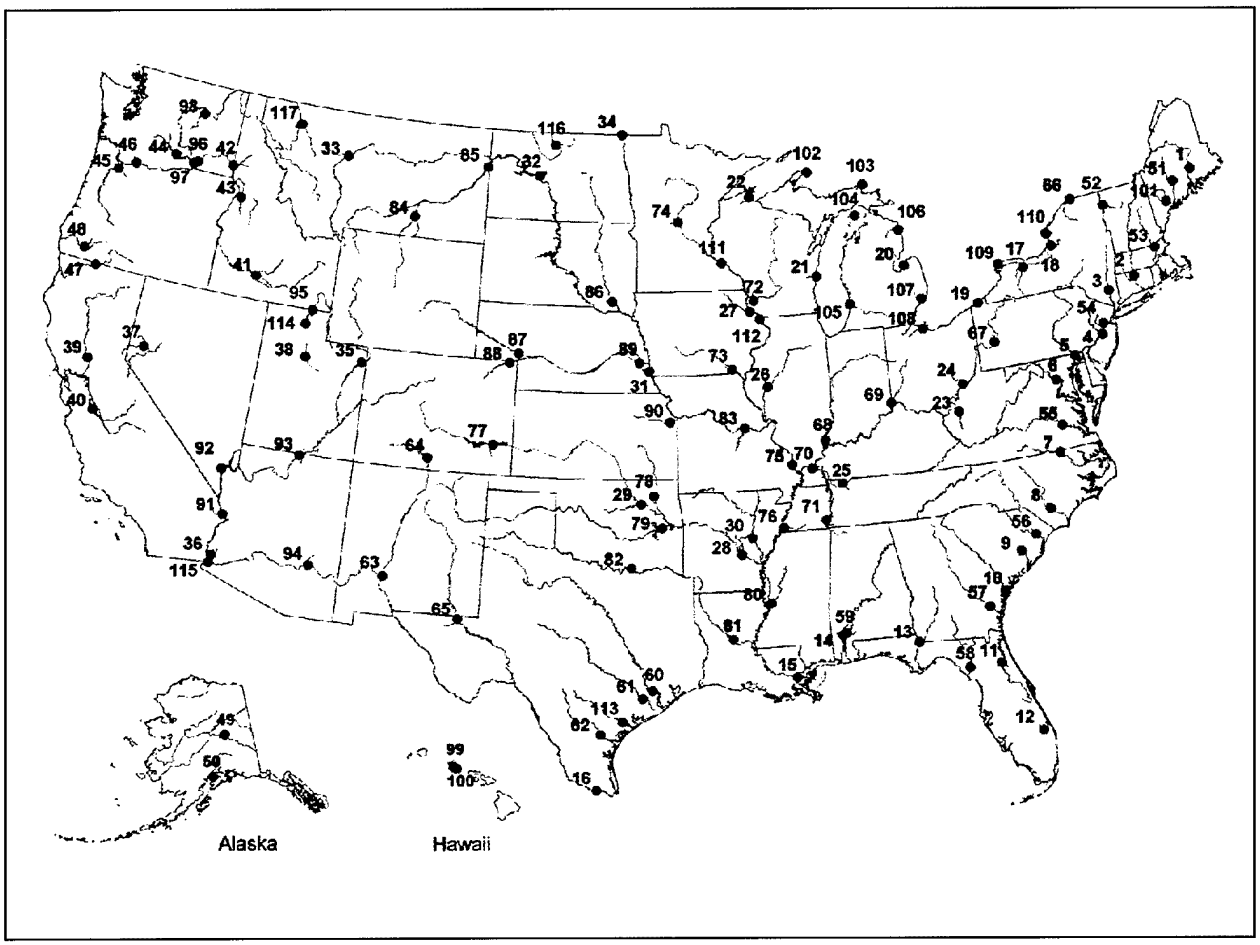


Figure A-1. The network of stations for the National Contaminant Biomonitoring Program.

Environmental Protection Agency (EPA). Concomitantly, the contaminant-related concerns of the FWS became more focused on the occurrence and effects of a larger array of contaminants in and on trust resources, especially those specific lands and organisms for which the FWS had received stewardship responsibility. These included the National Wildlife Refuge System; federally-listed threatened and endangered species, including the bald eagle (*Haliaeetus leucocephalis*); migratory birds, including waterfowl; and certain marine mammals, and anadromous and inter-jurisdictional fishes, such as Atlantic salmon (*Salmo salar*) and Great Lakes lake trout (*Salvelinus namaycush*). During the 1980's, contaminant concerns were further focused on Trust Resources by well-publicized wildlife kills, oils spills, and the acquisition of contaminated lands and other contaminant-related problems within the National Wildlife Refuge System (U.S. Fish and Wildlife Service 1986; U.S. General Accounting Office 1987; U.S. Department of the Interior 1990). The FWS believed that a comprehensive monitoring program would provide contemporaneous information on background contaminant and ecological conditions of trust resources, a necessary prerequisite to the documentation of contaminants and their effects.

Chemical Shortcomings

By the mid-1980's, many of the bioaccumulable contaminants that the NCBP and its predecessor were designed to monitor had been regulated, replaced, or both, and these changes were reflected by the fish and starling networks. As environmental concentrations of organochlorine chemical residues, mercury, and other contaminants declined in many media, concerns increased about the risks posed by other classes of chemicals, many of which had not been monitored under NCBP and were not amenable to traditional monitoring methods (Table A-1). Among these were short-lived, second- and third-generation pesticides, which were being used in large quantities in U.S. agriculture. These concerns resulted in part from increasingly frequent reports of fish and avian wildlife kills related to the use of highly toxic, but

short-lived, organophosphate, carbamate, and synthetic pyrethroid insecticides (Glaser 1995). In addition, the so-called soft pesticides are highly toxic to valuable non-target insects, including honey bees (Apidae) (Pimental et al. 1992). Herbicides such as atrazine were also being used in unprecedented amounts and had become widely distributed in the surface and ground waters draining agricultural areas (Paulson et al. 1993; Coupe et al. 1995). Herbicides have been identified as potential threats to important non-target plants, including the aquatic and terrestrial vegetation upon which wildlife depends (Kemp et al. 1983; Kemp et al. 1985; Fairchild et al. 1998). In addition, some widely used herbicides have been implicated as endocrine disruptors (Colborn et al. 1993; Goodbred et al. 1997) and others are either acutely toxic to aquatic organisms, contain toxic byproducts, or can be converted to toxic metabolites (e.g., Pothuluri et al. 1991).

In addition to agricultural chemicals, industrial and consumer chemicals not accounted for by the NCBP were also recognized as potential threats to fish and wildlife. For example, oil has long been a significant environmental pollutant (Schmitt 1999). In addition to the obvious direct toxic effects of oil spills, microliter quantities of oil are highly toxic to developing avian embryos (Albers 1977; Hoffman 1979; Hoffman 1990). Much of the toxicity of oil stems from the polycyclic aromatic hydrocarbons (PAHs) it contains. PAHs, which are created by the combustion of fossil fuels and by many industrial processes, are believed responsible for epizootics of tumors and other lesions in fish from many industrialized waterways (Malins et al. 1984; Baumann et al. 1991; Black and Baumann 1991). Like soft pesticides, PAHs and related compounds are difficult to monitor by traditional analytical methods. The most environmentally significant PAHs are either rapidly metabolized following their uptake by vertebrates (Baumann et al. 1982) or dissipate into the atmosphere, and chemical residues do not persist.

Several other classes of contaminants of potentially great environmental significance are difficult to monitor and assess by traditional analytical methods. Among these are phenols, alkylphenols, and diphenylalkanes, some of which have been identified as endocrine disruptors (Colborn et al. 1993). These groups include compounds such as phenol, a common and widespread industrial intermediate; cresol, a major component of creosote; the bisphenols (including bisphenol-A), which are components of most plastics (Perez et al. 1998); and the alkylphenols, which are surfactants discharged in large quantities from paper mills and sewage treatment facilities (Naylor et al. 1992; Nimrod and Benson 1996). Sewage discharges and agricultural activities also release large amounts of nutrients (i.e., nitrogen and phosphorous), which can stimulate nuisance blooms of algae and other microorganisms (such as *Pfisteria* spp. and *Protogoneaulax* spp.) that ultimately represent threats to human and ecological health (Rabalais et al. 1996; Gosselin et al. 1989; Kotak et al. 1993; Carmichael 1994; Burkholder et al. 1992; Burkholder et al. 1995). Bacteria can also convert excess nitrogen to ammonia, which can be acutely toxic to aquatic organisms (Wildhaber and Schmitt 1996). Munitions and chemicals associated with their manufacture contaminate some waterways and lands formerly associated with military activities. More recently, pharmaceuticals used in human and animal medicine have been found in public waterways and identified as environmental contaminants of potentially great significance (Steger-Hartmann et al. 1997; Hartmann et al. 1998; Halling-Sørensen et al. 1998; Buser et al. 1998).

Despite heightened concerns about new-generation pesticides and other classes of contaminants, questions about the ecological risks of regulated contaminants remain. Although environmental concentrations of many persistent elemental and organochlorine contaminants had declined by the 1980's, a growing body of information indicates that levels of these regulated contaminants are still sufficiently high in some areas to harm fish and wildlife in many important ecosystems, such as the Great Lakes (e.g., Colborn 1991). Organochlorine pesticide residues remain evident in some ecosystems where these compounds were used heavily in agriculture and in other applications, and in areas contaminated by sites of synthesis or formulation (Schmitt and Bunck 1995; Schmitt et al. 1999). Plasticizers such as di-n-butylpthatlate and bisphenol-A are ubiquitous environmental contaminants that can interfere with the control of reproduction by the endocrine system (Colborn and Clement 1992; Colborn et al. 1993; Perez et al. 1998). In addition, elemental contaminants (i.e., metals and metalloids) still constitute a threat from past and present mining activities, the use of organo-metallic pesticides and defoliants, and the continued growth of irrigated agriculture (Schmitt and Bunck 1995; Schmitt et al. 1999; Schmitt 1999). Lead remains a global pollutant from its historic (in the U.S.) and continued (elsewhere) use as a motor fuel and paint additive, and from mining, smelting, and other metallurgical activities (Settle and Patterson 1980; Nriagu 1990). Complex mixtures of polyhalogenated hydrocarbons (PHHs), such as PCBs, chlorinated dibenzo-*p*-dioxins (dioxins) and dibenzofurans (furans), and the insecti-

Table A-1. Contaminants of concern to the BEST Program (not mutually exclusive).

Contaminants	Common Mixtures, Congeners, Compounds, Elements	Use/Occurance	Biological Effects
PHHs			
Organochlorine pesticides	DDT and its metabolites, dieldrin, aldrin, endrin, mirex, chlordane, toxaphene, kelthane, HCB, BHC	Insecticides; fungicides; acaracides; rodenticides	Toxicity; endocrine disruption
PCBs	PCBs 77, 118, 126, 153, 169; Aroclors 1016, 1242, 1248, 1254, 1260	Dielectric, heat transfer, and hydraulic fluids; lubricants; plasticizers; copy papers	Cancer promoters; endocrine disruption; direct and developmental toxicity
Halogenated diphenyl ethers	Ugilec-141	PCB replacement compounds	Cancer promoters; endocrine disruption; direct and developmental toxicity
PCDDs, PCDFs	2,3,7,8-TCDD, 2,3,7,8-TCDF	Impurities; combustion products; chlorine bleaching process	Cancer promoters; endocrine disruption; direct and developmental toxicity
Phthalates	Di-n-butyl phthalate	Plasticizer	Toxicity; endocrine disruption
Elemental Contaminants			
Heavy metals Other toxic metals Metalloids	Hg, Cd, Pb Al, Sn As, Se	Natural constituents of the earth's crust; elevated concentrations occur as a result of mining and smelting; agriculture; metal manufacturing, fabrication, and finishing; pesticides and other chemicals; fossil fuel combustion	All can be toxic, but not necessarily at environmental concentrations; varies with medium
Herbicides	Atrazine, 2,4-D, Dacthal®, simazine, cyanazine, propanil	Most heavily-used class of agricultural chemicals in the U.S.	Contain toxic impurities and may degrade to toxic metabolites; endocrine disruptors; potentially toxic to non-target plants

Table A-1 continued.

Contaminants	Common Mixtures, Congeners, Compounds, Elements	Use/Occurance	Biological Effects
New-generation Pesticides			
Organophosphates Carbamates Synthetic pyrethroids	Parathion, diazinon Carbofuran, methyl carbamate Permethrin, esfenvalerate	Insecticides; fungicides	Avian and wildlife mortality; highly toxic to invertebrates and aquatic organisms
PAHs	Chrysene, benz(a)pyrene, benz(a)anthracene, naphthalene, fluorene	Present in petroleum creosote; formed from combustion of fossil fuels	Carcinogenic; teratogenic; mutagenic; some are highly toxic
Phenols			
	Nonylphenol, Polyethoxylates (APEs), Cresol, phenol	Surfactants; discharged from publicly-owned treatment works (POTWs) and paper or textile mills; creosote; industrial intermediates; plasticizers	Endocrine disruption
Diphenylalkanes			
Bisphenols	Bisphenol A	Plasticizer	Endocrine disruption
Pharmaceuticals			
Synthetic estrogens Antibiotics Analgesics Chemotheraputic agents Others	Ethenyl estradiol Fluoroquinelones Ibuprofen Cyclophosphamide Clofibric acid	Used in human and veterinary medicine; detected in sewage treatment plant effluents and receiving waters	Endocrine disruption
Munitions	Nitro-aromatic explosives and derivatives (TNT, DNT), white P, RDX	Military installations; production, testing, and use of weapons/explosives	Highly toxic; mutagenic
Nutrients and biotoxins	Nitrogen, phosphorus	Agriculture (fertilizers, manure); sewage; manufacture of explosives	Stimulate nuisance growths of algae and other aquatic plants, cyanobacteria, and dinoflagellates that produce biotoxins and cause anoxia; can be converted to highly toxic compounds (nitrogen to ammonia, sulfur to hydrogen sulfide)
Hydrogen ions	Mostly sulfuric acid	Sulfur (as SO_2) released to the atmosphere from the combustion of fossil fuels forms sulfuric acid, which is responsible for acid rain. Acid mine drainage from coal and metals mining has also resulted in significant biological consequences.	Highly toxic; significant changes in community composition; pH and related variables are important modulators of contaminant toxicity, especially for metals.

cides toxaphene and chlordane, are especially problematic. The components of these mixtures differ greatly in toxicity and persistence, and there is evidence that some of the most toxic constituents and metabolites are also among the most resistant to degradation (Gooch and Matsamura 1987; Tillitt et al. 1992). Moreover, no large-scale monitoring program has yet incorporated the periodic assessment of the dioxins and furans owing to the great expense and difficulty of their analysis. Local-, regional-, and national-scale investigations have shown that these and other highly toxic and accumulative PHHs, as well as other structurally and toxicologically similar compounds, are present in many U.S. ecosystems at concentrations either actually or potentially harmful to fish and wildlife (Colborn 1991; Kubiak et al. 1989; Keuhl et al. 1989; Tillitt et al. 1992; Mac and Edsall 1991; U.S. Environmental Protection Agency 1992). In addition, mercury concentrations in some remote areas are rising (Monteiro and Furness 1998), ostensibly from the atmospheric transport of mercury released during the combustion of coal (U.S. Environmental Protection Agency 1997). For these reasons, concentrations of bioaccumulative contaminants in fish have been proposed as an indicator of sustainable economic development by the U.S. Council on Environmental Quality (U.S. Council on Environmental Quality 1997) and remains an important component of most environmental monitoring programs (e.g., Hirsch et al. 1988; Messer et al. 1991).

Features of the BEST Program

The intent of the BEST program is to build on the success of the NCBP while addressing some of its identified shortcomings by monitoring for a broad array of contaminants and their effects and framing the findings in terms of Department of the Interior (DOI) issues. Because of the large number of contaminants not amenable to monitoring by traditional analytical methods, the basic premise of measuring only chemical concentrations in selected environmental media is now perceived as inadequate. In addition, chemical concentrations alone are not useful for assessing the cumulative effects of multiple contaminants or the cumulative effects of contaminants and other factors such as sedimentation, eutrophication, and disease. It is widely known that contaminants and other stressors tend to co-occur as a consequence of land-use and industrial practices (e.g., Schmitt 1999). Assessing the cumulative effects of multiple contaminants and differentiating contaminant-induced effects from those caused by other factors, information that is necessary to initiate and evaluate management actions, requires more than just the concentrations of a relatively small number of contaminants in habitats and organisms.

Weight-of-Evidence Approach

The BEST program documents temporal and spatial trends in the exposure of organisms and ecosystems to contaminants and the effects of exposure on selected organisms through the application of chemical and biological monitoring methods. The monitoring methods comprise scientifically well-founded and relatively inexpensive techniques chosen to maximize the number of samples that can be analyzed and, therefore, the number of sites that can be monitored. The results of these monitoring activities may stimulate research targeted towards confirming and diagnosing the nature, extent, and significance of the findings using more robust, sensitive, and expensive methods; however, this research is beyond the scope of the BEST program. Through this approach, the program hopes to overcome some of the geographic and chemical shortcomings of the NCBP by sampling a wider variety of habitats using screening methods that are sensitive to many classes of chemicals, and restricting the application of more definitive methods to focused, follow-up research. "Workhorse" methods that can be applied to many taxa and guilds of organisms in a wide variety of habitats, and at spatial scales ranging from local to regional and national, are employed. By using methods consistently across ecosystems, guilds, and spatial scales, the findings can be aggregated for the analysis and interpretation of spatial and temporal trends. Unlike the NCBP, which relied solely on the measurement of contaminant concentrations in fish and wildlife, the BEST program incorporates complementary biological methods spanning many levels of biological organization, as well as information from extant sources. This strategy, referred to collectively as a *weight-of-evidence approach*, represents a holistic, integrated assessment of the interactions between physical and chemical factors and living organisms. The National Water Quality Assessment Program of the USGS Water Resources Division (Hirsch et al. 1988) and the Environmental Monitoring and Assessment Program of the U.S. EPA (Messer et al. 1991), both of which evolved contemporaneously with the BEST program, are also based on weight-of-evidence approaches. So

is the Sediment Quality Triad (Chapman et al. 1991; Chapman 1992), a procedure in which sediments are evaluated through chemical analysis, toxicity testing, and benthic macroinvertebrate community composition. Through the application of this approach and the minimization of redundancies among the methods employed, the BEST program can monitor a wide array of habitats and ecosystems for many classes of contaminants in an efficient and cost-effective manner.

Organisms and Habitats Sampled

To remedy some of the taxonomic and programmatic deficiencies of the NCBP, the BEST program bases its measurements on ecosystems, habitats, and organisms of interest to DOI agencies. Sampling of both organisms and habitats ensures that the exposure of mobile species (especially birds and fishes) is documented. For chemical analyses and the application of organism- and population-level methods, guilds of organisms were selected for sampling on the basis of the following criteria:

(1) They have a high potential for exposure and a measurable response (including the accumulation of tissue residues) to contaminants of concern.

(2) They have a territory or home range that overlaps the area to be monitored.

(3) They are sufficiently large and abundant to permit easy enumeration and sampling.

(4) They represent, either in actuality or as a surrogate, a guild or species of programmatic importance to the DOI.

Guilds, which are groups of ecologically similar taxa, were selected in lieu of particular species because no fishes or birds that meet the above requirements have distributions that cover all lands or waterways monitored under the BEST program. The guilds have been selected in such a manner as to ensure that the maximum number of chemical classes and methods can be accommodated with the smallest number of taxa and, hence, collections and analyses. The identification of guilds rather than species also allows substitutions for species of programmatic importance to DOI agencies in the event that a species of concern cannot be sampled [i.e., ospreys (*Pandion haliaetus*) or cormorants (*Phalacrocorax* spp) substituted for eagles; common carp (*Cyprinus carpio*) for rare cyprinids; rainbow trout (*Oncorhynchus mykiss*) for rare salmonids].

The guilds selected for sampling have been chosen to ensure that all classes of bioaccumulable contaminants will be accounted for should they occur, but that the BEST program will also be sensitive to the occurrence of other contaminant classes that might not accumulate. Two pairs of guilds—primary and secondary—have been identified for the application of analytical chemistry, biomarker, and population health indicators in aquatic and estuarine habitats (Table A-2), with the secondary guild to be sampled in the event that no taxa representing the primary guilds are present at a site.

Table A-2. Primary and secondary guilds identified for sampling aquatic habitats. From BEST Program (1996).

Primary guild	Secondary guild	Major contam. classes
Piscivorous bird	Piscivorous fish	Bio-accum.[1]
Sediment-dwelling fish	Filter-feeding invertebrate	PAH, other non-bio-accum.[2]

[1]PHHs, mercury, selenium, arsenic

[2]PAHs and elemental contaminants that do not bioaccumulate; and sediment-bound organic compounds not accounted for analytically

These guilds were selected because of the wealth of information that continues to show that piscivorous fishes and wildlife remain at greatest risk to contaminants and their effects in aquatic ecosystems. Because of the recognized importance of the PAHs as environmental pollutants and the fact that most toxic PAHs are rapidly metabolized by vertebrates and do not accumulate, sediment-dwelling organisms are included in both levels of the matrix. Other classes of contaminants can be accounted for by sampling different media and by other methods, such as toxicity tests and community analyses.

The aquatic habitats selected for sampling by the BEST program have been classified hierarchically (Table A-3). The classes were selected primarily on the basis of the guilds of organisms likely to be found

and the methods that would be used to sample and test them (BEST 1996).

Table A-3. Habitat classification system used to identify habitats for sampling. From BEST Program (1996).

Estuarine		
	Open water	
	Vegetated (wetland)	
Freshwater		
	Flowing	
		Large river (i.e., not wadeable)
		Wadeable stream
	Standing	
		Open water
		Vegetated wetland
Marine		
(open water only habitat considered)		

Monitoring Methods

The BEST program employs a suite of monitoring and assessment methods chosen to address many classes of contaminants and their effects. The suite includes traditional chemical analyses of selected media (animal carcasses, sediments) for contaminants that persist sufficiently long and accumulate to concentrations that permit their periodic measurement at reasonable cost; and biological indicators spanning many levels of biological organization — from molecular through population and community — that integrate the effects of multiple contaminants and other stressors. The biological indicators can also document the exposure of organisms and ecosystems to new or previously unrecognized chemicals as well as chemicals for which analytical methods are either not currently available or that are too expensive to be incorporated into a large monitoring program.m.

Both general and specific indicators have been selected so that the BEST program can provide a degree of early-warning and can assess the cause(s) of biological effects. Methods specific to individual contaminants or specific classes of contaminants are more sensitive and respond more rapidly than general methods, and can consequently provide a warning of impending or incipient problems from particular contaminants. In addition, their specificity is of obvious value for the assessment of causation. Conversely, general methods can provide information about contaminants for which specific methods either do not exist or are not useful in a monitoring context. General methods such as those indicative of organism, population, or community health can also be used to assess the cumulative effects of multiple contaminants and the combined effects of contaminants and other factors such as sedimentation, eutrophication, and disease. Such methods may be the most effective way to monitor the exposure of organisms or ecosystems to ephemeral contaminants.

To meet its stated objectives, the BEST program considers methods acceptable if they meet the following criteria, which were derived from the DOI's Damage Assessment Guidance Document (U.S. Department of the Interior 1987):

1. The method is well documented in the peer-reviewed scientific literature.

2. The method has been shown to be well correlated with contaminants, other stressors, or both across a gradient of concentrations — both in the laboratory and in the field — and has been shown to yield reproducible and verifiable results.s.

3. The method has been used to a great enough extent that data are available with which to statistically document its performance.

4. Given 3, the method must be able to detect relevant differences (between sites, time-period, taxa, etc.) at the desired level of significance ($P < 0.05$) with financially realistic sample sizes and sampling frequencies.

5. Confounding or interfering variables that affect the performance of the method are known, and the intended application of the method takes these variables into account.

6. The application of the method has been documented in the scientific literature for the species, medium, habitat, guild, or community for which its use is proposed.

The specific monitoring and assessment methods being evaluated for use in aquatic habitats by the BEST program have been drawn from three main categories: analytical chemistry, toxicity tests and bioassays, and biomarkers and indicators of organism health. The BEST program also uses structural and functional indicators of population and community

health, which are being evaluated through partnerships and leveraging with other investigations and programs. Within each of these categories, methods were proposed by working groups comprising experts from the U.S. Geological Survey, other agencies, universities, and the private sector (BEST 1996). Attributes of the categories are summarized here.

Analytical Chemistry

Assessing concentrations of toxic chemicals in environmental media and how their distribution changes over time and space is the most common environmental monitoring technique. Although it is particularly useful for those classes of contaminants that bioaccumulate or have long residence times, analytical chemistry alone cannot provide information on contaminant exposure and effect, for a number of reasons. First, analyte concentrations in abiotic media may not accurately portray risk because of bioavailability constraints. Consequently, the measurement of total metals or organic chemical residue concentrations in some media—especially soils and sediments—may have little relation to actual or even potential toxicity. Similarly, contaminant concentrations in biotic media do not necessarily provide direct measures of toxicity because contaminants may be sequestered in fats or other tissues, stored as non-toxic forms, or otherwise immobilized and rendered non-toxic. Contaminants may also be incorporated in recalcitrant tissues (bone, scales, etc.) not amenable to digestion, or may occur as solid-phase material either on the outside of organisms (Schmitt and Finger 1987) or inside, as gut contents (Brumbaugh and Kane 1985). These materials may not be harmful to either the organism itself or to higher trophic level organisms.

Although there is a growing body of information with which to assess the cumulative effects of some classes of contaminants (e.g., Sprague and Ramsay 1965; Enserink et al. 1991; Ahlborg 1989; van den Berg et al. 1998) and complex mixtures in sediments (Long and Morgan 1991; Long et al. 1995; Swartz et al. 1995; Ingersoll et al. 1996; Wildhaber and Schmitt 1996; Wildhaber and Schmitt 1998), the ecological risk assessment (U.S. Environmental Protection Agency 1992) of chemical residue data remains centered largely at the level of the individual contaminant. Due to differing requirements for sample handling and storage, the suite of analytes to be considered must be pre-determined, which presumes forehand knowledge of contaminants of concern. The availability of analytical standards also tends to limit analyses to parent compounds and their most abundant and persistent metabolites. Unfortunately, the number of analytes for which methods and standards are available represents only a small fraction of the potentially toxic commercial and industrial substances — as well as their precursors, metabolites, and byproducts — released into the environment. Despite these limitations, analytical chemistry remains attractive and necessary for three primary reasons: (1) The sensitivity and relative precision of most analytical chemistry enables the detection and periodic quantification of analytes at sublethal levels in a variety of media, which provides important information for managing and regulating impending or incipient problems; (2) As noted previously, the quantification of contaminants in prey species and various environmental media provides information with which to assess risk, albeit for individual or small numbers of contaminants; and (3) In combination with other evidence, analytical chemistry forms the basis of cause-effect analysis and risk assessment.

Bioassays and Toxicity Tests

Bioassays and toxicity tests provide direct evidence of cumulative contaminant effects on the survival, growth, behavior, or reproduction of living organisms while controlling for extraneous confounding factors. The results of such tests can also be extrapolated to the population and community level and can be used to differentiate contaminant effects from those induced by other perturbations. Additionally, toxicity tests and bioassays can provide cost-effective, rapid assessments of cumulative toxicity, albeit at the expense of chemical specificity. Toxicity tests and bioassays can be performed *in vitro*, with cells or tissues from a variety of organisms (Fabacher 1982; Tillitt et al. 1991; Zabel et al. 1995), or *in vivo*, with whole organisms ranging in size from bacteria (Johnson 1998; Johnson and Long 1998) to vertebrates (Finger and Bulak 1988; Hall et al. 1993). Endpoints can range from a biochemical signal of pollutant exposure or genetic damage (Fabacher 1982; Tillitt et al. 1991; Johnson 1998) to the death or loss of motility of organisms in a short-term test (Finger and Bulak 1988; Hall et al. 1993; Wildhaber and Schmitt 1996). Bioassays and toxicity tests, especially those conducted with whole organisms, are typically quite general with respect to the contaminants eliciting the response; however, they can also provide more specific information on the nature and identity of the substances involved when multiple tests are conducted with organisms of differing susceptibility to certain contaminants (Ingersoll et al. 1992; Wildhaber and Schmitt 1996). Greater resolution can also be obtained when toxicity tests are combined

with reductionist approaches such as Toxicity Identification and Evaluation (Mount 1988; Mount and Anderson-Carnahan 1988) or by selectively sampling (Huckins et al. 1993; Johnson 1998; Johnson and Long 1998) or fractionating (Tessier et al. 1979; Tessier et al. 1984; Hansen et al. 1996) the test medium either prior to testing or after.

Biomarkers and Indicators of Organism Health

Biomarkers include biochemical, physiological, morphological, and histopathological responses of organisms signifying chemical exposure (Melancon 1995). Biomarkers can sometimes be used to estimate or predict chemical exposure, effects, or both at higher levels of organization, thereby enabling evaluation of cause-effect linkages between environmental exposure and biological responses (Kloepper-Sams et al. 1994). At the molecular level, biomarkers can provide early warning of potential higher level effects that may not be obtainable through chemical analyses or other methods of investigation. Some indicators are especially useful for documenting exposure to and the effects of ephemeral toxins as well as those that do not accumulate in environmental media. Although some are specific, many biomarkers are quite general. Responses can be unique to one contaminant or a relatively small group of structurally similar chemicals, or they may be general indicators of organism, population, community, or ecosystem health that respond to a wide variety of chemical and other stressors.

Biomarkers are attractive alternatives to more traditional measures because they integrate the cumulative effects of adverse conditions at the suborganismal level to indicate whether any precede whole-organism or population-level effects (Adams 1990). Specific biomarkers are useful diagnostic tools for assessing cause-effect relationships. In addition, because the toxicological response to a chemical is caused by the interaction between the toxicant and a cellular or extracellular component (e.g. protein, nucleic acid), these responses may occur before any are observed at higher levels of organization. As noted by Mayer et al. (1992), the most useful biomarkers are those that are closely related to individual fitness (survival, growth and reproduction) because they represent potential surrogates for population-level indicators.

Population and Community Health Indicators

Although not being evaluated in the ongoing projects (Schmitt et al. 1995; Bartish et al. 1997), population- and community-level responses and indicators constitute an important part of the BEST program. Indices of population and community structure and function reflect differences in the relative abundance, size, biomass, or age of organisms at different points in space and time, and can integrate the effects of multiple stressors across individuals and species. Structural metrics have long been recognized as being among the most sensitive indicators of environmental quality, and have a rich history in the assessment and regulation of water quality (Washington 1984). Because most population- and community-level indicators are non-specific and may consequently yield little information regarding the nature of the stress, they are often used together with other, more specific indicators such as environmental chemistry and toxicity tests. Some community-level metrics, such as the Ephemeroptera-Plecoptera-Trichoptera index (Plafkin et al. 1989), Hilsenhoff's (Hilsenhoff 1987) and Lenat's (Lenat 1993) benthic macroinvertebrate biotic indices, and the Index of Biotic Integrity (Karr 1981; Karr et al. 1986; Karr et al. 1987; Bramblett and Fausch 1991) for fishes, reflect the differential susceptibilities of certain taxa to contaminants and other stressors. At the population level, the documentation of growth, reproduction, and survival rates of selected fishes, birds, and mammals can be important, albeit general, indicators of contaminant exposure. Population- and community-level indicators, indices and metrics are based on the relative abundance and size of organisms in taxon and age groups. As such, they largely reflect injury that has already occurred, but by integrating the effects of a wide variety of contaminants and stressors they are important for environmental monitoring.

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13. ABSTRACT (*Maximum 200 words*)
This document describes the suite of biological methods of the U.S. Geological Survey-Biomonitoring of Environmental Status and Trends program for monitoring chemical contaminants and their effects on fish. The methods, which were selected by panels of experts, are being field-tested in rivers of the Mississippi River, Columbia River, and Rio Grande basins. General health biomarkers include a health assessment index based on gross observation; histopathological examination of selected organs and tissues; condition factor; and the heptosomatic and splenosomatic indices. Immune system indicators are plasma lysozyme activity and measures of splenic macrophage aggregates. Reproductive biomarkers include plasma concentrations of sex steroids hormones (17β–estradiol and 11-ketotestosterone) and vitellogenin, gonadal histopathology (indluding reproductive stage and, in females, gonadal atresia), and the gonadosomatic index. Indicators of exposure to polycyclic aromatic and polyhalogenated hydrocarbons are the H4IIE rat hepatoma cell bioassay (performed on solvent extracts of composite fish samples) and hepatic ethoxyresorufin-*O*-deethylase activity. Stable nitrogen isotope ratios are used to assess the trophic position of the fish and their exposure to sewage and other animal wastes. For each indicator we describe endpoints(s) and methods, and discuss the indicator's value and limitations for contaminant monitoring and assessment.

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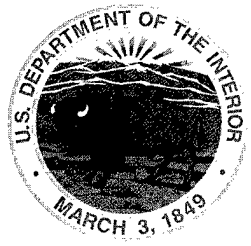

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